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The Four Ribosomal DNA Units of the Malaria Parasite *Plasmodium berghei*

IDENTIFICATION, RESTRICTION MAP, AND COPY NUMBER ANALYSIS*

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The four ribosomal RNA genes (rDNA units) of the rodent malaria parasite, *Plasmodium berghei*, were identified and mapped by restriction enzyme analysis and Southern blot hybridization of genomic DNA. Although the four genes share common characteristics, they appear to be internally different from each other in xpanse and sequence. One *Hind*III site near the 3′ end of the coding region for the large rRNA is the only restriction site which we have detected that is conserved in all of the genes. The distance between the conserved *Hind*III site and the coding region for the small rRNA is at least 1–2 kilobases longer in two of th rDNA units than in a third unit. None of the four rDNA units were linked by restriction mapping where about 150 kilobases of the genome were accounted for. The copy number of two of the rDNA units was determined to be approximately 1 per haploid genome by quantitative analysis of cloned (plasmid) DNA *versus* genomic DNA digests on Southern blots. The other two gen s also appear to be present in 1 copy. Restriction analysis confirms both the low copy number and that these genes are not in an easily recognizable tandem array. The low number of rDNA units requires that n w copies of the genome produced during intraerythrocytic growth be active in RNA synthesis soon after their replication.

Malaria, which continues to be a major world health problem, is caused by intraerythrocytic, protozoan parasites of the genus *Plasmodium*. The complete life cycle of these organisms involves growth in both a vertebrate and an invertebrate host and presents numerous morphologically defined developmental forms. Molecular biology offers approaches to the understanding and eventual control of this disease, but at present, relatively little is known about any species of *Plasmodium* at the molecular level. The DNA content of these organisms is small, reported to be about 0.02–0.05 pg/nucleus (1, 2), and has a very low G[1] + C content, ~20% (3).

We have selected the rodent malaria, *Plasmodium berghei*, as a model system to study the genome of this unusual class of organisms. The first gene family we have studied is the rRNA gene family. In a previous report, we characterized the major rRNA species and a clone of a single rDNA unit (4). The polarity of the gene, 5′-small rRNA-large rRNA-3′ is the same as that for rDNA units of all other organisms studied. The intact large rRNA is about 4,000 nucleotides long and is cleaved *in vivo* near the 5′ end into two fragments, about 3,100 and 900 nucleotides long. The small rRNA is about 2,200 nucleotides long.

In this paper, we report that there are only four rDNA units in the haploid genome. The four rDNA units have different restriction maps and have no apparent tandem arrangement. Evidence is also presented to show that they are not amplified during the intraerythrocytic asexual stage of the life cycle. This indicates that, of the organisms which do not amplify their DNA, *P. berghei* has fewer copies of these classic dosage repeat genes than any other eukaryote (5).

EXPERIMENTAL PROCEDURES

Growth and Preparation of Parasites—The NYU2 and NK65 strains of *P. berghei* were grown in white mice by passage of infected blood. Strain NK65 was periodically passed via *Anopheles stephensi* to retain its ability to produce gametocytes. Parasitized blood was collected, depleted of leukocytes and platelets, and saponin lysed, as described previously (4).

Extraction of Nucleic Acid—DNA was routinely extracted from saponin-treated NYU2 strain parasites with SDS, incubated with proteinase K, and banded in the presence of ethidium bromide on a CsCl gradient (4). DNA was also prepared after treatment with SDS and proteinase K by extracting once with an equal volume of phenol (saturated with 50 mM Tris, 10 mM EDTA, pH 7.5), once with chloroform:phenol:isoamyl alcohol (25:24:1, v/v/v) and twice with chloroform:isoamyl alcohol (24:1, v/v). Pancreatic RNase, heat-treated to destroy DNase activity, was added to 0.1 mg/ml, incubated at 37 °C for 15 min and re-extracted as above. The DNA was precipitated in 70% ethanol, 0.1 M sodium acetate, pH 6.5, overnight at −20 °C and collected by centrifugation at 10,000 rpm in an SS-34 rotor at −20 °C for 20 min. Total RNA was extracted by phenol (4). DNA was prepared from each plasmid clone as a supercoil on CsCl gradients containing ethidium bromide (6).

Southern Blot Analysis—After restriction enzyme digestion, DNA fragments were electrophoresed at 2.25 V/cm for 14 h through 0.8% agarose gels (4). Nitrocellulose blots were prepared as described by Southern (7) and hybridized as described previously (4). DNA probes were labeled by nick translation with [α-^{32}P]dCTP (8). RNA probes were ^{32}P-labeled in the 5′ position with [γ-^{32}P]ATP after partial hydrolysis in 0.1 M sodium borate, pH 9.3, at 65 °C using polynucleotide kinase (4). Autoradiograms of hybridized blots were prepared using X-Omat AR film exposed at −70 °C with the use of an intensifying screen. Materials and enzymes were described previously (4).

Sucrose Gradients—*P. berghei* NYU2 DNA (200 μg) was digested with *Eco*RI (2 units/μg) or *Hind*III (2 units/μg) for 2.5 h at 37 °C and the reaction stopped by adding EDTA to a final concentration of 50 mM. The DNA fragments were then applied to a 10–40% sucrose gradient and centrifuged at 25,000 rpm for 18 h at 20 °C as described by Maniatis (9). Each gradient was fractionated from the bottom. The DNA in each fraction was precipitated in 70% ethanol, washed with 80% ethanol, dried, and resuspended in 60 μl of 1 mM EDTA, pH 8.0. Aliquots of 5 μl were sufficient for Southern blot analysis.

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[1] The abbreviations used are: G, guanine; C, cytosine; rDNA unit, ribosomal DNA unit; SDS, sodium dodecyl sulfate; kb, kilobase, bp, base pair.

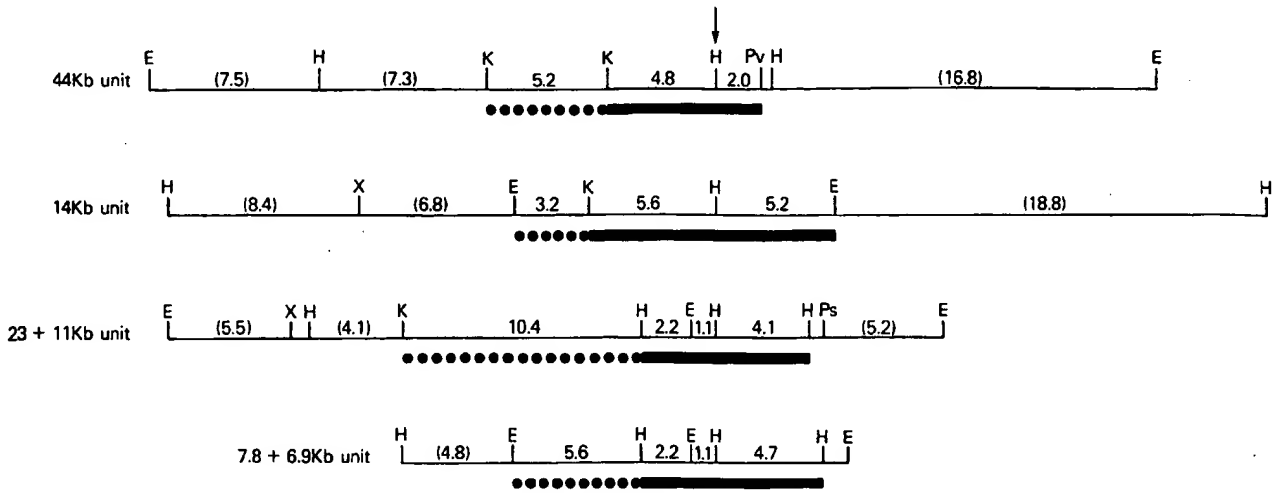

FIG. 4. **Restriction map of the rDNA of P. berghei.** Restriction maps of 4 distinct rDNA units, the 44 kb, 14 kb, 23 + 11 kb, and 7.8 + 6.9 kb units are presented. (*E*, *Eco*RI; *H*, *Hind*III; *K*, *Kpn*I; *Pv*, *Pvu*II; *Ps*, *Pst*I; and *X*, *Xba*I). The distances between restriction sites are in kilobases. Those distances bounded by parentheses were calculated from the size differences between two or more fragments and are therefore less accurate than those directly measured. The smallest lengths of DNA shown to contain complete small rRNA and large rRNA coding regions are indicated with the symbols, ● and ▬, respectively. Maps are drawn 5′ → 3′ with respect to the RNA transcript.

Diphenylamine Assay—Diphenylamine assays were performed essentially by the method of Ashwell (10). Duplicate samples of stock DNA solutions containing approximately 10 μg of DNA as judged by A_{260} were brought to a volume of 340 μl with 10 mM Tris, 1 mM EDTA, pH 8.0, and 660 μl of diphenylamine reagent added. The samples were heated for 10 min in capped tubes at 100 °C. Absorbance at 595 and 650 nm was determined and the difference taken. A salmon sperm DNA stock solution prepared by weight was used to prepare standards, 5 to 50 μg, which were assayed simultaneously.

RESULTS[2]

Restriction Mapping with Genomic DNA—Southern blot analysis of *P. berghei* DNA revealed multiple bands hybridizing to the large and/or small rRNA (4) suggesting that there were several different rDNA units. We have mapped four separate units containing coding regions for both large and small rRNAs. Since phage clones of all rDNA fragments were not available (4), restriction mapping was done with genomic DNA using *Eco*RI and *Hind*III restriction sites as a framework. *Eco*RI-and *Hind*III-digested genomic DNA fragments were separated according to size by ultracentrifugation through continuous 10–40% sucrose gradients. Southern blots, prepared from each fraction of the two gradients and from a portion of each fraction digested with the other enzyme (*Hind*III or *Eco*RI, respectively), were hybridized with total RNA probe (Figs. 1 and 2). The number of the gradient fraction in which each rDNA band is at its maximal concentration was determined by visual analysis of the autoradiograms. This number defines the *Eco*RI and *Hind*III fragments which give rise to each *Eco*RI + *Hind*III fragment (Figs. 1 and 2). Using the four rDNA clones described in Fig. 3*A*, each of which contains sequences homologous to different portions of the small and large rRNAs, the ribosomal coding sequences in each *Eco*RI, *Hind*III, and *Eco*RI + *Hind*III fragment were characterized (Fig. 3*B*). The relationship between the *Eco*RI and *Hind*III fragments defined in Figs. 1 and 2, together with the hybridization properties of each of the fragments in Fig. 3*B*, allows us to outline the maps of the four different rDNA units shown in Fig. 4. Details of the mapping procedure and data used to construct the maps of the four rDNA units are presented in the mini print section.

Four Nonidentical rDNA Units—The restriction sites surrounding the coding sequences of the rDNA units differ, and conservation of the three central restriction sites of the 23 + 11 kb and the 7.8 + 6.9 kb rDNA units represents the most extensive homology between any of the rDNA units detected by restriction analysis. The *Hind*III site designated in Fig. 4 with an arrow, however, appears to be conserved in all four rDNA units. The clone of the 3′ end of the coding region of the 23 + 11 kb unit, pPbL4.1, hybridizes only to sequences beyond this *Hind*III site in the other 3 units, and the clone, pPbL1.1, which is adjacent and 5′ to the sequence in pPbL4.1 in the 23 + 11 kb unit does not. Mapping with *Eco*RV (4) indicates that the 3′ end of the small rRNA gene in the 7.8 + 6.9 kb unit lies near the *Hind*III site bounding the 5.6 kb *Eco*RI/*Hind*III fragment. With the various coding regions localized relative to *Eco*RI, *Hind*III, and *Kpn*I restriction sites for all four of the rDNA units, it was apparent that the 14 kb and 44 kb rDNA units have a greater distance between the conserved *Hind*III site in the large rRNA gene and the 3′ end of the coding region for the small rRNA than is present in the 7.8 + 6.9 kb unit. The difference (1–2 kb or more) suggests that either the spacer between the two rRNA genes is larger in these rDNA units or that an intron is present in the large rRNA gene.

Copy Number Analysis—The number of copies of two of the *P. berghei* rDNA units, the 7.8 + 6.9 kb and the 23 + 11 kb units was estimated by measuring the amounts of the 7.8 kb *Eco*RI fragment, 5.6 kb *Eco*RI/*Hind*III fragment, 4.1 kb *Hind*III fragment, and the 3.3 kb *Hind*III fragment present in genomic DNA by comparison with carefully measured quantities of cloned rDNA from pPbSL7.8, pPbS5.6, pPbL4.1, and pPbL3.3. Plasmid pPbSL7.8 derived from the 7.8 + 6.9 kb unit contains the insert in pPbS5.6 plus that in pPbL2.2;

[2] Portions of this paper (including part of "Results," Fig. 1–3, 5, and 6 and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-2673, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

and plasmid pPbL3.3, also from the 7.8 kb + 6.9 kb unit, contains the inserts in pPbL2.2 and pPbL1.1 (4). The DNA content of genomic DNA and supercoiled plasmid DNA stock solutions was determined by diphenylamine assay. Genomic and plasmid DNAs were digested with the appropriate enzymes, and six concentrations of digested genomic and plasmid DNA, 62.5 to 2000 ng and 31.25 to 1000 pg, respectively, were applied with carrier tRNA to agarose gels. Following electrophoresis, Southern blots were prepared and hybridized with nick translated probe pPbSL7.8, pPbS5.6, pPbL4.1, or pPbL3.3, respectively. Autoradiograms of those blots are shown in Fig. 7*A*. The appropriate band was excised from each well of each blot and counted *versus* a blank prepared from an adjacent nonhybridizing piece of the blot. The cpm in each band are plotted *versus* the amount of DNA applied to the gel in Fig. 7*B*. The plots of genomic DNA are nearly linear in each case over the full range of amounts of DNA applied. The plots of the plasmid DNA were also linear over the lower part of the range, but in the cases of pPbSL7.8, pPbS5.6, and pPbL4.1, each became nonlinear above about 250 to 500 pg. By comparing the plasmid and genomic DNA curves within the linear range, it was possible to determine the amount of the rDNA band of interest present in a measured amount of genomic DNA. Assuming the genome size of *P. berghei* to be $\sim 5 \times 10^7$ bp from the measured DNA content (0.05 pg) per nucleus (2), the number of copies/genome of each of the four rDNA bands was calculated. Those values were 1.0, 1.5, 1.0, and 2.8 copies/haploid genome for the 7.8 kb *Eco*RI fragment, the 5.6 kb *Eco*RI/*Hind*III fragment, the 4.1 kb *Hind*III fragment, and the 3.3 kb *Hind*III fragment, respectively. These values indicate that there is about one copy of the 7.8 + 6.9 kb rDNA unit and one copy of the 23 + 11 kb rDNA unit. Further, it appears to be possible to extend the approximation of about 1 copy/genome to the 44 kb rDNA

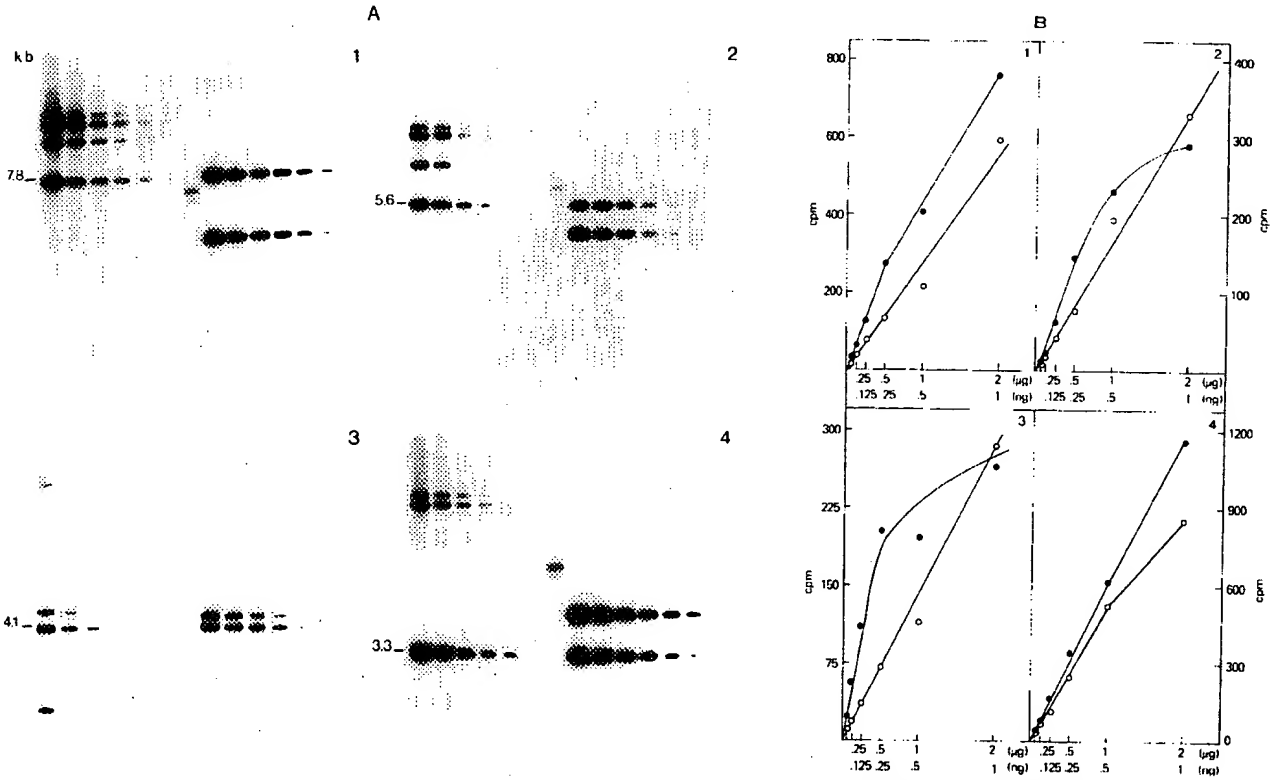

FIG. 7. **Quantitation of rDNA copy number by Southern blot analysis.** *A*, *1*, aliquots (10 μg) of *P. berghei* DNA and pPbSL7.8 supercoiled plasmid DNA from stock solutions, assayed immediately prior to use by diphenylamine assay, were digested with *Eco*RI and diluted to 50 ng/μl with 10 mM Tris, 1 mM EDTA, pH 8. Aliquots of the *P. berghei* DNA solution (40, 20, 10, and 5 μl) and aliquots (25 and 12.5 μl) of a 1/10 dilution (to 5 ng/μl) in electrophoresis buffer containing 0.1 μg/μl of yeast tRNA were mixed with 10 μl of 0.5 mg/ml of yeast tRNA, 10 μl 15% Ficoll, 0.01% bromphenol blue and brought to a volume of 50 μl with H_2O and applied to the 0.8% agarose gel in *lanes 1–6*, respectively. A *Hind*III digest of λ DNA was applied in *lane 7* as a size standard. Aliquots (20, 10, and 5 μl) of the digested plasmid DNA diluted 1/1000 (to 50 pg/μl) in electrophoresis buffer containing 0.1 μg/μl of yeast tRNA, and aliquots (25, 12.5, and 6.25 μl) of a further 1/10 dilution (to 5 pg/μl) in the same buffer plus tRNA were mixed with 10 μl of 0.5 μg/μl of yeast tRNA, 10 μl 15% Ficoll, 0.01% bromphenol blue and brought to a volume of 50 μl with H_2O and applied to the 0.8% agarose gel in lanes *8–13*, respectively. After electrophoresis, a Southern blot was prepared and hybridized with pPbSL7.8 probe. An autoradiogram of the hybridized blot is shown. *2*, aliquots of *P. berghei* DNA and pPbS5.6 supercoiled plasmid DNA were analyzed as described in *1* except that the DNAs were digested with *Eco*RI + *Hind*III, and pPbS5.6 probe was hybridized with the blot. *3*, aliquots of *P. berghei* DNA and pPbL4.1 supercoiled plasmid DNA were analyzed as described in *1* except that the DNAs were digested with *Hind*III, and pPbL4.1 probe was hybridized with the blot. *4*, aliquots of *P. berghei* DNA and pPbL3.3 supercoiled plasmid DNA were analyzed as described in *1* except that the DNAs were digested with *Hind*III, and pPbL3.3 probe was hybridized with the blot. *B*, using the autoradiograms shown in *A* as a guide, the genomic band corresponding to the plasmid insert band, the plasmid insert band, and suitable blanks were excised from each well of each blot and counted in 10 ml of liquid scintillation cocktail for 20 min. A plot of cpm *versus* the amount of total DNA applied to the gel is presented for: *1*, pPbSL7.8, 7.8 kb *Eco*RI fragment; *2*, pPbS5.6, 5.6 kb *Eco*RI/*Hind*III fragment; *3*, pPbL4.1, 4.1 kb *Hind*III fragment; *4*, pPbL3.3, 3.3 kb *Hind*III fragment.

unit and the 14 kb rDNA unit by comparing the hybridization of total RNA probe to a blot of a *Hind*III + *Eco*RI digest of genomic DNA (Fig. 8*A*), since the 2.4 and 5.2 kb fragments of the 44 and 14 kb rDNA units, respectively, hybridize with the probe about as well as the 4.7 and 4.1 kb fragments which arise from single copy rDNA units.

The restriction analysis is consistent with both the low copy number and the fact that these genes are not in an easily recognizable tandem array. Individual genes have been mapped for between 5 and 20 kb in either direction beyond their ribosomal coding regions and no common areas have been detected (Fig. 4). Restriction enzymes *Pst*I, *Xba*I, and *Pvu*II, which cut only outside the coding expanse of the rDNA units, all yield 3 or 4 large but defined fragments covering 150 kb minimally (Fig. 8*B*). This indicates that, if each gene is repeated, more than 150 kb of DNA is involved and that the sites for these enzymes which are outside the rRNA coding areas are well preserved. Since we can find no bounds or repeated units, it is impossible for us to say how much DNA is involved with the ribosomal gene structure.

Absence of Extrachromosomal rDNA—Given the low number of copies of the rDNA units detected, the DNA of the organism was examined for extrachromosomal copies of rDNA which may have been lost during DNA preparation by our standard procedure. Saponin-treated parasites were prepared, lysed in SDS, and the DNA extracted with phenol and precipitated. Genomic DNA prepared by this procedure and the standard procedure were assayed by the diphenylamine assay and equal amounts of undigested and *Eco*RI-digested DNA of each were compared by Southern blot hybridization with total RNA probe (Fig. 9). Undigested DNA from both preparations lack discrete DNA bands either by ethidium bromide staining (not shown) or by blot hybridization. Southern blot analysis of *Eco*RI-digested DNA from the two preparations indicate that the same amount of rDNA is present per μg of DNA from both methods of preparation. These findings allow the conclusion that *P. berghei* contains four independent rDNA units which are not extrachromosomally amplified during asexual intraerythrocytic growth.

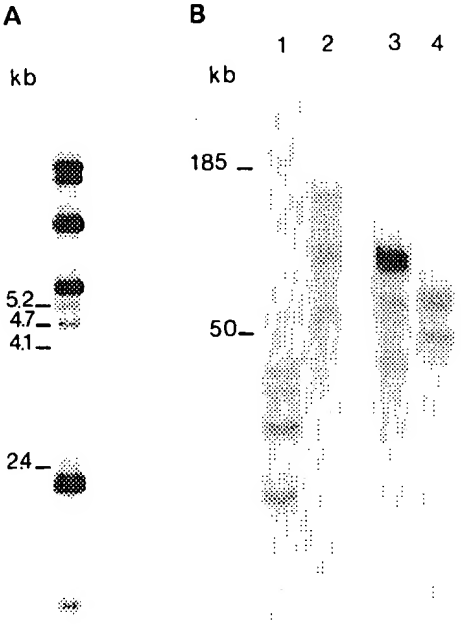

FIG. 8. **Copy number and proximity of the four rDNA units.** *A*, extrapolation of copy number estimate to other rDNA units. A Southern blot prepared from 2 μg of *Eco*RI + *Hind*III-digested *P. berghei* DNA electrophoresed through a 0.8% agarose gel was hybridized with total RNA probe. The sizes of the rDNA fragments homologous to the 3′ end of the large rRNA are indicated on the autoradiogram shown. *B*, hybridization analysis of large rDNA fragments. Aliquots (1 μg) of genomic *P. berghei* DNA were digested with *Xba*I, *Pst*I, *Bam*HI, and *Pvu*II and electrophoresed on a 0.2% agarose gel formed on gel bond (FMC Corp., Marine Colloids Division) at 1.5 v/cm for 108 h in *lanes 1–4*, respectively, with undigested λ phage and vaccinia virus DNA as size standards. After ethidium staining and photography, the gel was dried at 37 °C onto the gel bond. The dried gel was treated in alkaline solution to denature the DNA, neutralized as described for the blotting of agarose gels (6), and then instead of blotting, used directly for hybridization to total RNA probe as described for nitrocellulose blots.

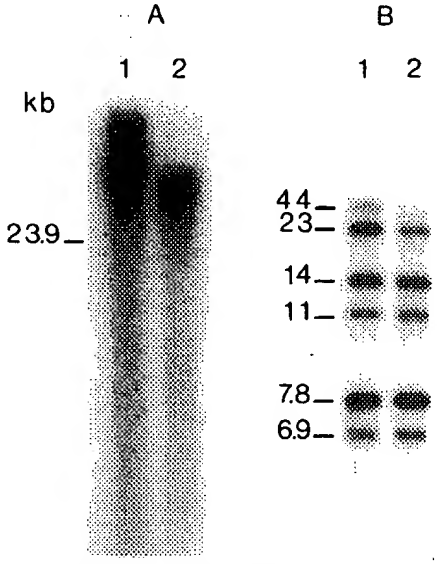

FIG. 9. **Assessment of amplification of rDNA in asexual erythrocytic stage parasites.** *A*, a Southern blot prepared from 2-μg samples of undigested *P. berghei* DNA prepared on CsCl (*lane 1*) and by phenol extraction (*lane 2*) electrophoresed through a 0.5% agarose gel was hybridized with total RNA probe. An autoradiogram of that blot is shown. *B*, an autoradiogram is shown of a Southern blot prepared and hybridized as described for *A* except that the DNA was digested prior to electrophoresis with *Eco*RI, and that a 0.8% agarose gel was used.

DISCUSSION

We have presented data which indicate that *P. berghei* has four rDNA units. Although they share some common characteristics, they appear to be internally different from each other in expanse and sequence. The only feature of the restriction maps which we have detected that is common to all four rDNA units is a *Hind*III restriction site near the 3′ end of the coding sequence for the large rRNA. Differences among the units were detected from the location of *Eco*RI, *Hind*III, and *Kpn*I restriction sites internal to the transcribed regions. Further, mapping data show that the distance between the conserved *Hind*III site in the large rRNA gene and the end of the coding region for the small rRNA is longer in the 44 and 14 kb units than in the 7.8 + 6.9 kb unit (4). This indicates either that an intron is present in the large rRNA gene or that the transcribed spacer is larger in those two units. This implies that different precursor ribosomal rRNAs are processed during ribosomal maturation if both types of genes are transcribed.

None of the four genes were linked by restriction mapping where about 150 kg of the genome was accounted for; therefore, it is not possible to say how the rDNA units are located relative to each other. They may be clustered over several

hundred kb or spaced widely in the genome. This arrangement is unlike most other eukaryotic organisms which have a large number of genes in a tandem arrangement. Rather the arrangement appears to be more like that of *Escherichia coli* where the rDNA units, although clustered in certain places on the chromosome, are well spaced, and apparently independent coding units (11).

Genes encoding rRNAs are thought to exhibit dosage repetition or amplification in order to produce enough of their gene product in the appropriate time to satisfy protein biosynthetic requirements of the cell (12). As an example, the protozoan *Tetrahymena*, which has a germinal, micronucleus containing only one rRNA gene copy (13), amplifies its rRNA gene in the macronucleus during growth to about 600 copies/haploid genome (14). Since we have been unable to detect amplification of rDNA in the asexual blood stages, *P. berghei* with only four has fewer rDNA units/haploid genome than any other eukaryote (5). Based on an approximation of the rate of rRNA synthesis calculated as described (15, 16), it is, however, plausible that the four (or fewer functioning) rDNA units/genome are capable of producing sufficient rRNA for cell growth if each of the genomes generated during the intraerythrocytic cycle are active in rRNA synthesis from the time of their genesis. Assuming that *Plasmodium* RNA polymerases function approximately as eukaryotic polymerases and transcribe at 83 nucleotides/s (17, 18) and pack at a density of 100 polymerase molecules/rDNA unit (19), four rRNA transcription units 12.5 kb in length could produce the rRNA for 2.7 ribosomes each second. Over the 24 h growth cycle, 2.3×10^5 ribosomes could be generated, and since ribosomes are stable (20), almost all would accumulate. It is believed (21, 22) that the parasite contains about 0.25×10^{-12} g of RNA/haploid genome; most is present as rRNA (4) representing about 0.65×10^5 ribosomes. A fully developed, segmenting *P. berghei* schizont in a mouse erythrocyte contains an average of 8 nuclei (23) and, therefore, about 5×10^5 ribosomes. In order for this number of ribosomes to accumulate, an average of two (or more) genomes (*i.e.* ~8 rDNA units) must be active for the equivalent of the 24-h growth cycle. Considering that the results of several investigators indicate that DNA replication in *Plasmodium* is initiated early and continues through much of the intraerythrocytic growth cycle (21, 23, 24), and further, that the rate of RNA synthesis also continually increases up to segmentation (25), our data and calculations indicate that the newly replicated copies of the genome are active in rRNA synthesis.

In most eukaryotes, rRNA is synthesized and processed in a nucleolus. No distinct nucleolus has been found in primate or rodent malarias (26). This difference is probably related to the low copy number of rRNA genes. In contrast, avian malarias appear to have a compact nucleolus (26); it would be of some interest to know whether avian malarias have a higher copy number of rRNA genes.

Our data indicate that at least two classes of rDNA units are present in different locations in the genome. The above calculations show that even if only one class with two rDNA units functions in the intraerythrocytic stage, it is still possible to produce the required number of ribosomes, provided an equivalent of ~4 genomes are active over the 24-h cycle. Further, it appears that during its history this organism has responded to different selective pressures in the vertebrate and invertebrate host since *P. berghei* exhibits different highly specialized cellular forms intracellularly in the rodent at 37 °C and extracellularly in the mosquito at 18 °C. It is reasonable to consider that the functioning of the *Plasmodium* ribosome may have been subject to different sets of selection pressures producing two different types of ribosomes. Alternatively, it is possible that different parts of the genome function during the various growth stages and synthesize similar ribosomes from different loci. Thus, differences in the rDNA might arise by functional separation. It will be of interest to determine whether or not expression of the rDNA is differentially controlled through the life cycle.

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Supplementary Material to "The Four Ribosomal DNA Units of the Malaria Parasite Plasmodium berghei: Identification, Restriction Map, and Copy Number Analysis

John B. Dame and Thomas F. McCutchan

Identification of the 23 + 11kb rDNA unit

Four Eco RI fragments appear to be arranged in pairs and constitute two similar rDNA units. The 23 kb + 11 kb and the 7.8 kb + 6.9 kb Eco RI fragments contain rDNA units which by restriction analysis contain a similar central portion. In the experiment shown in Fig. 1, the 2.2 kb and 1.1 kb Eco RI/Hind III fragments, which contain the central portion of the rDNA, each peak in two different fractions; the 23 kb and 7.8 kb Eco RI fragments contain the 2.2 kb fragment, and the 11 kb and 6.9 kb Eco RI fragments contain the 1.1 kb fragment. These two Eco RI/Hind III fragments were both derived solely from the 3.3 kb Hind III fragment by Eco RI digestion (Fig. 2) indicating that Eco RI fragments giving rise to them were present in the genome as pairs. Since the 7.8 + 6.9 kb unit was cloned in phage λ (4), we conclude that the 23 kb and 11 kb Eco RI fragments are also paired forming an rDNA unit. As indicated in Fig. 4, the region of the rDNA coding for the 3' end of the large rRNA is in a 4.7 kb Hind III fragment from the 7.8 + 6.9 kb rDNA unit and in a 4.1 kb Hind III fragment from the 23 + 11 kb rDNA unit. Also, the coding region for the small rRNA is within a 14.5 kb Hind III fragment of the 23 kb Eco RI fragment which is larger than the comparable fragment (10.4 kb) from the 7.8 + 6.9 kb unit.

Mapping the 44kb and 14 kb rDNA units with EcoRI and Hind III

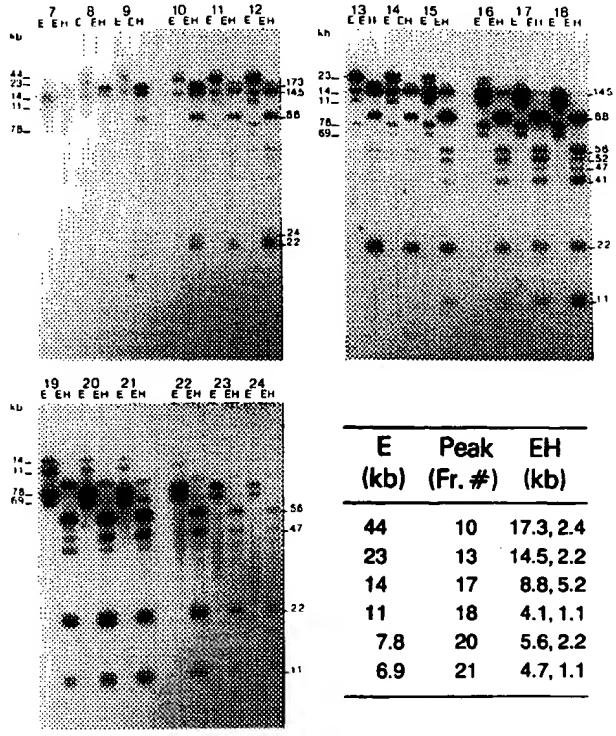
The remaining two of the six Eco RI fragments, the 44 kb and 14 kb fragments, contain sequences which hybridize to both small and large rRNA probes indicating that they both have at least one set of rDNA sequences. Digestion of the 44 kb Eco RI fragment with Hind III produces a 17.3 kb and a 2.4 kb fragment which hybridize to DNA. The 17.3 kb fragment hybridizes to pPbS5.6, pPbL2.2, and pPbL1.1 indicating that it contains the coding area for the small rRNA and the 5' end of the large rRNA. The 2.4 kb fragment hybridizes to pPbL4.1 which is homologous to the 3' terminal portion of the large rRNA. Accurately positioning the Hind III sites within the 44 kb Eco RI fragment and thereby establishing that there is a single contiguous rDNA unit present required further analysis of the fragment by digestion with Kpn I (see below). Hind III cleaves the 14 kb Eco RI rDNA unit yielding two fragments 8.8 kb and 5.2 kb in length as indicated in Fig. 2. Hybridization of the 8.8 kb fragment to pPbS5.6, pPbL2.2 and pPbL1.1 indicates that this fragment contains the same region of the ribosomal gene found in the 17.3 kb fragment of the 44 kb Eco RI rDNA unit. The 5.2 kb fragment like the 2.4 kb fragment hybridizes only to pPbL4.1 which is homologous to the 3' end of the large rRNA. The 14 kb Eco RI fragment therefore contains a single rDNA unit arranged as illustrated in Fig. 4.

Analysis with Kpn I

Kpn I cleaves genomic DNA at five places within the four rDNA units and their surrounding sequences as indicated in Fig. 4. In order to locate the Kpn I sites within the rDNA units, DNA from fractions of the sucrose gradient of Eco RI fragments (Fig. 1) was digested with Kpn I. Sites were found only within the 44 kb, 23 kb and 14 kb Eco RI rDNA fragments. The sizes of the Kpn I and Kpn I/Eco RI fragments derived from each Eco RI rDNA fragment which hybridize with total RNA probe are summarized in Table I. To order the Kpn I sites relative to the coding regions of each rDNA unit, Southern blots of Kpn I + Eco RI, Kpn I + Hind III and Kpn I digested genomic DNA were hybridized to rDNA clones and the type of coding region in each fragment identified as shown in Fig. 5 and summarized in Table I. Kpn I sites are located in the 44 kb, 14 kb and 23 + 11 kb rDNA units as shown in Fig. 4 and each further defines the location of the regions homologous to the small rRNA in these units. The size and hybridization properties of the Kpn I + Hind III fragments were consistent with these assignments (Fig. 4, Table I); however, in order to draw the map of the 14 kb rDNA unit, it was necessary to postulate the existence of two different ~24 kb Hind III fragments which together contained this rDNA unit. To prove this, DNA from the peak fraction from Fig. 2 containing the 24 kb Hind III fragment(s) was digested with Kpn I. A 24 kb fragment which hybridizes only to pPbL4.1 and two fragments, 18.5 kb and 5.6 kb which hybridize to pPbSL7.8 (contains inserts found in pPbS5.6 and pPbL2.2) are generated (Fig. 6). The 5.6 kb Kpn I/Hind III fragment hybridizes with pPbL2.2 and pPbL1.1 probe and the 18.5 kb Hind III fragment hybridizes with pPbS5.6 probe (Fig. 5). These results indicate that two different 24 kb Hind III fragments contains segments of the 14 kb Eco RI rRNA unit. One contains the coding areas for the small and most of the large rRNA gene and is cleaved by Kpn I, and the other contains the 3' terminus of the large rRNA gene and is not cleaved by Kpn I.

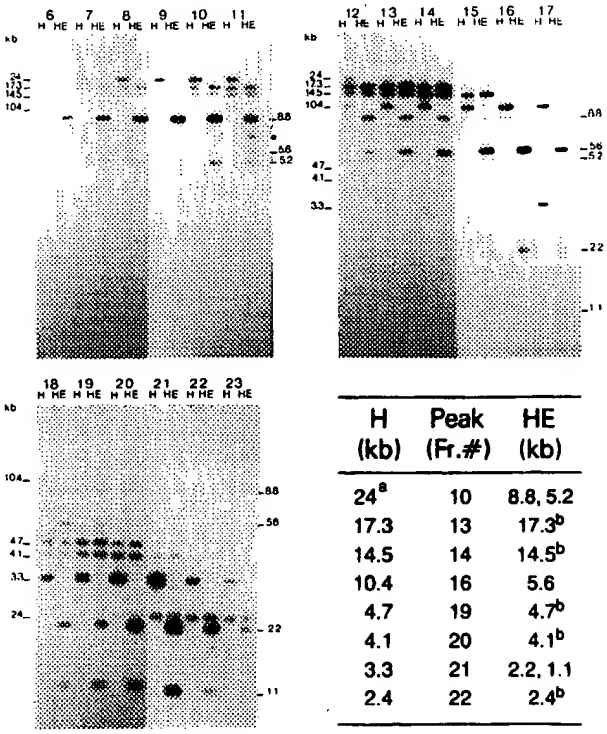
Analysis with Pvu II, XbaI and Pst I

Pvu II, Xba I and Pst I each make a single cut in only one Eco RI rDNA fragment, Pvu II (44 kb), Xba I (23 kb) and Pst I (11 kb). A Pvu II + Kpn I digest yields a 6.8 kb fragment from the 44 kb Eco RI rDNA unit which hybridizes to the probes homologous to the complete large rRNA gene. A Pvu II + Hind III digest yields a 2.0 kb fragment which hybridizes to pPbL4.1, the probe for the 3' terminal portion of the large rRNA gene, thus placing the site (Fig. 4). An Xba I digest of the 23 kb Eco RI fragment yields a 17.5 kb fragment which hybridizes to rRNA placing the site as indicated (Fig. 4). Digests with Xba I + Kpn I and Xba I + Hind III placed an Xba I site in the non-coding region flanking the 14 kb Eco RI fragment (Fig. 4) yielding 10 kb and 15.6 kb fragments, respectively, which hybridize to RNA probe. The 10 kb Xba I/Kpn I fragment hybridized only to pPbS5.6, homologous to the small rRNA gene. A Pst I + Eco RI digest of the 11 kb Eco RI fragment yields a 5.8 kb rDNA fragment thereby placing the Pst I site (Fig. 4).



E (kb)	Peak (Fr. #)	EH (kb)
44	10	17.3, 2.4
23	13	14.5, 2.2
14	17	8.8, 5.2
11	18	4.1, 1.1
7.8	20	5.6, 2.2
6.9	21	4.7, 1.1

Fig. 1. Analysis of Eco RI rDNA fragments by sucrose gradient fractionation and double digestion with Hind III. Genomic DNA was digested with Eco RI and fractionated over a 10-40% sucrose gradient. Aliquots of each fraction digested with the indicated enzymes were electrophoresed through 0.8% agarose gels and Southern blots prepared. Blots were hybridized to total RNA probe prepared using RNA from strain NK65. Autoradiograms of blots containing DNA from fractions 7 through 24 are shown. The sizes of Eco RI fragments hybridizing in each panel are indicated on the left; the size of Eco RI + Hind III fragments are indicated on the right. The fraction in which each fragment is at its highest concentration is indicated in the figure summary table. The Eco RI fragment giving rise to each Eco RI + Hind III fragment is defined by the fraction number. E = Eco RI, EH = Eco RI + Hind III.



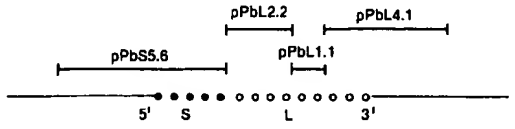
H (kb)	Peak (Fr.#)	HE (kb)
24[a]	10	8.8, 5.2
17.3	13	17.3[b]
14.5	14	14.5[b]
10.4	16	5.6
4.7	19	4.7[b]
4.1	20	4.1[b]
3.3	21	2.2, 1.1
2.4	22	2.4[b]

Fig. 2. Analysis of Hind III rDNA fragments by sucrose gradient fractionation and double digestion with Eco RI. Genomic DNA was digested with Hind III and fractionated over a 10-40% sucrose gradient. Aliquots of each fraction, digested with the indicated enzymes were electrophoresed through 0.8% agarose gels and Southern blots prepared. Blots were hybridized to total RNA probe prepared using RNA from strain NK65. Autoradiograms of blots containing DNA from fractions 6 through 23 are shown. The sizes of Hind III fragments hybridizing in each panel are indicated on the left; the size of Hind III + Eco RI fragments are indicated on the right. The fraction in which each fragment is at its highest concentration is indicated in the figure summary table. The Hind III fragment giving rise to each Eco RI + Hind III fragment is defined by the fraction number. H = Hind III, HE = Hind III + Eco RI.

[a] Contains two different fragments which appear as a single band, see Fig. 6.

[b] Not cleaved by Eco RI.

A



B

Probe	EcoR1 Fragment (Kb)	HindIII Fragment (Kb)	EcoR1 + HindIII Fragment (Kb)
pPbS5.6	44, 23, 14, 7.8	24, 17.3, 14.5, 10.4	17.3, 14.5, 8.8, 5.6
pPbL2.2	44, 23, 14, 7.8	24, 17.3, 3.3	17.3, 8.8, 2.2
pPbL1.1	44, 14, 11, 6.9	24, 17.3, 3.3	17.3, 8.8, 1.1
pPbL4.1	44, 14, 11, 6.9	24, 4.7, 4.1, 2.4	5.2, 4.7, 4.1, 2.4

Fig. 3. Analysis of Eco R1, Hind III, and Eco R1 + Hind III rDNA fragments with plasmid subclones homologous to specific regions of the large and small rRNAs. A. Clones pPbS5.6, pPbL2.2, and pPbL1.1 were derived from a clone of the 7.8 + 6.9 kb rDNA unit in Charon 4A, λPbR27, and clone pPbL4.1 was derived by direct cloning from genomic DNA (4). One or more of these probes were homologous to each rDNA fragment which was detectable in a genomic digest with total RNA probe, and none of the probes cross hybridize with each other. Their properties are summarized by the stylized drawing shown. (••• coding sequence small rRNA; ooo coding sequence large rRNA).

B. Southern blots prepared from Eco R1, Hind III and Eco R1 + Hind III digested P. berghei DNA electrophoresed through 0.6% agarose gels were hybridized to nick translated probes prepared from pPbS5.6, pPbL2.2, pPbL1.1, and pPbL4.1. Bands in each digest hybridized by the probe indicated are recorded by size.

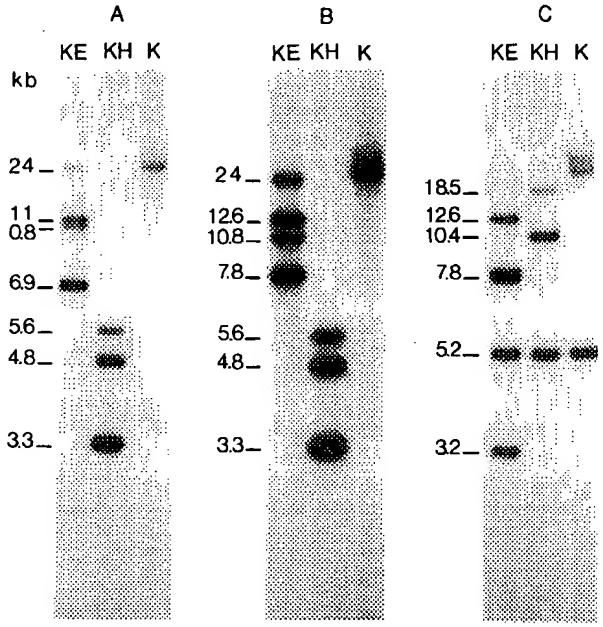


Fig. 5. Analysis of Kpn I, Kpn I/Eco R1 and Kpn I/Hind III rDNA fragments with probes homologous to specific regions of the large and small rRNAs. Southern blots were prepared from 1 µg samples of P. berghei DNA digested with Kpn I + Eco R1 (KE), Kpn I + Hind III (KH) and Kpn I (K), electrophoresed on a 0.8% agarose gel and hybridized to pPbL1.1 (A), pPbL2.2 (B) and pPbS5.6 (C) probes. An autoradiogram of each is shown in the figure with the sizes of all fragments 24 kb and smaller indicated on the left.

TABLE I

Kpn I fragments of rDNA units

rDNA Unit	Digests		
	Kpn I	Kpn I + Eco R1	Kpn I + Hind III
44 kb	5.2 (S)[a,b]	24(L)	4.8(L)
23 + 11 kb		12.6(S+L)	10.4(S)
14 kb		3.2(S)	5.6(L)
		10.8(L)	18.4(S)

[a] Fragment size in kilobases.

[b] Homology to plasmid clones of the coding region of the small rRNA (S) or the large rRNA (L).

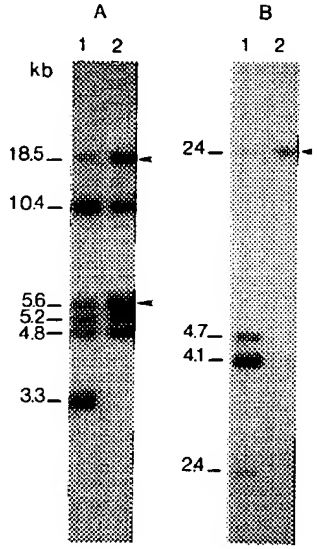


Fig. 6. Detection of two 24 kb Hind III rDNA fragments comprising portions of the 14 kb rDNA unit. Southern blots were prepared from a 2 µg sample of P. berghei DNA digested with Hind III + Kpn I (lane 1) and 5 µl of fraction 10 of the Hind III digested DNA described in Fig. 2 digested with Kpn I (lane 2) after electrophoresis through a 0.8% agarose gel. The blot used to prepare the autoradiogram shown in panel A was hybridized with pPbSL7.8 probe, and that in panel B with pPbL4.1 probe. The sizes of all bands are given on the left, and bands which are enhanced in the fractionated DNA samples are marked with arrows on the right.

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FURTHER STUDIES AND ELECTRON MICROSCOPIC CHARACTERIZATION OF *PLASMODIUM BERGHEI* DNA

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The average length and the interspersion pattern of repetitive DNA sequences in the *Plasmodium berghei* genome have been studied by electron microscopy. Within the limitations posed by the relatively high genome complexity, analysis of partially renatured total DNA indicates that repetitive sequences do not occupy preferential positions along the genome, but are widely dispersed (one in approx. 8000 base pairs of unique DNA). Structures appearing as loops flanked by inverted repeats are present. Analysis of the repetitive fraction purified by hydroxyapatite chromatography shows that the average length of rapidly reassociating repetitive structures is around 800 base pairs with 90% of the length distribution between 400 and 1400 base pairs. Suitable extraction methods, preserving circularity of extrachromosomal DNA components, allow the detection of molecules which can be identified as mitochondrial DNA, 10.5 ± 0.4 μm long.

Key words: *Plasmodium berghei*; Repetitive DNA; Interspersion pattern; Mitochondrial DNA

INTRODUCTION

Previous studies [1, 2] concerning physico-chemical properties and informational content of *P. berghei* DNA revealed a measurable difference in the ratio of repetitive to unique DNA associated with variations in infectivity toward mosquitoes.

The evidence from biological and physico-chemical experiments indicates that variations in genome organization involving the amplification of some genome portions are associated with the production of viable gametocytes. Thus, when infectivity is high, repetitive sequences amount to 16–18% of total DNA extracted from intraerythrocytic population; when infectivity is low (or in the extreme case, zero) similar preparations from the same strain contain not more than 3–5% repetitive DNA.

In order to investigate further the validity of the above hypothesis, it is important to obtain a better characterization of the repetitive sequences, especially as regards their

Abbreviations: PB, phosphate buffer; TE buffer, 0.1 M Tris-HCl, 0.01 M EDTA, pH 8.6.

length and their distribution along the genome. It would also be of value to detect and characterize the mitochondrial DNA component.

The present work reports the results obtained by electron microscopic analysis of different DNA preparations extracted from the fully infective NK 65 strain, namely total DNA, its repetitive fraction purified by hydroxyapatite chromatography, and partially purified mitochondrial DNA, the objective being to understand the role of repetitious DNA in gametogenesis.

MATERIALS AND METHODS

Strain. Strain NK 65 freshly passed through mosquitoes was obtained from the Wellcome Research Laboratories through the courtesy of Dr. Victoria Latter. It was stored by us in liquid nitrogen. Thawed samples were fully infective for the mosquito for at least 50 passages in mice.

Various procedures. For the preparation and purification of plasmodia, DNA extraction, sonication, sedimentation velocity and renaturation kinetics see ref. 2.

Hydroxyapatite (HAP) chromatography. The method described by Britten et al. [3] was followed. HAP (DNA grade Biogel HTP) was purchased from BioRad. Columns containing 1 ml of packed material, equilibrated at 60°C with 0.12 M phosphate buffer, were loaded with 0.9 ml of sample containing 20–30 μg of sonicated DNA, denatured and renatured to C_0t value corresponding to a renatured fraction of about 13% as judged from spectrophotometric measurement. In order to have practicable C_0t values (approx. 5 min) actual renaturation was carried out (at 60°C) on 0.3 ml samples containing 20–30 μg (C_0 approx. 70–100 μg ml^{-1}) of sonicated and denatured DNA in 0.36 M phosphate buffer; once the desired C_0t value was obtained, the partially renatured samples were diluted three times with warm distilled water and loaded directly onto the columns. Elution with 0.12 M phosphate buffer (PB) (8 fractions of 1 ml each) was followed by elution with 0.4 M PB (6 fractions, 1 ml each). The DNA content of each fraction was determined spectrophotometrically. Fractions containing reassociated (repetitive) DNA (i.e. the first 2 or 3 fractions eluted at 0.4 M PB) were pooled, dialysed against 0.3 M sodium acetate, precipitated with two volumes of absolute ethanol at –20°C, and stored at this temperature until use.

Electron microscopy. Mounting of DNA samples for electron microscopy was performed according to Kleinschmidt's technique as modified by Davis et al. [4] (formamide spreading). 35 μl of the hyperphase containing 25% formamide, 1 μg ml^{-1} DNA and 100 μg ml^{-1} cytochrome *c* in TE buffer (0.1 M Tris-HCl, 0.01 M EDTA, pH 8.6) were spread onto the hypophase (distilled water) in a 10 cm Petri dish. Copper grids 200–300 mesh, covered with a thin collodion film, were used. After touching the cytochrome monolayer, grids were dehydrated in 95% ethanol, stained in an alcoholic

solution of uranyl acetate, and again dehydrated in 95% ethanol, following which the specimens were rotary shadowed with Pt in an Edwards apparatus (model 12E6/1707). A Siemens Elmiskop I electron microscope was used at direct enlargement 8000–40 000 times. Micrographs were taken on Kodak Electron Image 4463 (6 × 9) film.

Length measurements on electron micrographs and determination of linear density. Individual molecular images were measured on enlarged prints of electron micrographs with a digitizer Tektronix 4956, connected to a Tektronix computer, kindly made accessible to us by Dr. M. Bongiorno-Nardelli at the Institute of Histology and Embryology of the University of Rome. Length measurements, reduced for the total enlargement factor (direct enlargement × photographic enlargement) were translated into molecular weights (expressed in daltons or in base pairs) by multiplying by the proper value of the linear density. These values for single stranded and double stranded DNA were determined by parallel measurements on φX174 viral DNA (Miles Biochemicals; M_r 1.77×10^6 daltons [5]) and on PBR 322 plasmid (Boehringer Mannheim Biochemicals; M_r 2.88×10^6 daltons [6]). With our conditions they turned out to be 1.27×10^6 daltons $\mu m^{-1} = 3.85$ kb μm^{-1} and 2.15×10^6 daltons $\mu m^{-1} = 3.25$ kb μm^{-1}, respectively.

Mitochondrial DNA extraction. A modified version of the procedure described by Kilejian [7] was followed. Purified plasmodia were resuspended in a buffered sucrose solution (0.3 M sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) and homogenised with an Ultra-Turrax apparatus. Homogenization was performed in two steps, 30 s each, at half of maximum power. The homogenate, diluted with an equal volume of the buffered sucrose solution, was centrifuged at 2000 rpm for 5 min several times until no precipitate was detectable, so as to eliminate large debris and nuclei. The resulting suspension was then spun for 10 min at 10000 rpm, to pellet mitochondria. This pellet was resuspended in 10 mM Tris-HCl (pH 8.0), 30 mM NaCl, 20 mM EDTA, 2% SDS and lysis was accomplished for 20 min at 37°C. The lysate was then treated with T1 RNAase 100 U ml^{-1} for 30 min at 37°C, then with proteinase K, 100 μg ml^{-1} for 1 h at 37°C. After enzyme digestion, a suitable volume of a 4 M NaCl solution was added to make a final concentration of 0.5 M. DNA was extracted by three treatments with chloroform-isoamyl alcohol (24:1, v/v). Subsequently ethidium bromide (final concentration 350 μg ml^{-1}) and CsCl (final refractive index $n_D^{(25°C)} = 1.3870$) were added and the resulting solution was spun for 24 h at 36000 rpm using a SW 50.1 rotor in a L8/80 Beckman Spinco ultracentrifuge. Fractions were collected by puncturing the bottom of the tube. The DNA containing fractions were identified with a mineral-light UV lamp in the dark, pooled and finally dialysed against 0.3 M sodium acetate.

RESULTS

Electron microscopic observation of repetitive sequences in total DNA. Electron microscopic observation of partially renatured DNA molecules allows, in principle, the determination of both length distribution and interspersion pattern of rapidly reassociating repetitive sequences. Complete mapping of repetitive stretches in the genome of *P. berghei* certainly goes beyond the capability of the method, since the genetic complexity of the organism (1.0×10^{10} daltons [1]) corresponds to a physical genome length greater than 1.5×10^4 kbases (approx. 5 mm), while extracted DNA in normal preparations has a molecular weight of 15×10^6 daltons (approx. 24 kbases, or 7.5 μm). Nevertheless, direct observation of such DNA fragments after denaturation and partial reassociation is useful in revealing the type of repetitive structures present.

To this aim, a suitable C_0t value has been chosen, corresponding to almost full reassociation of the rapidly renaturing component with a negligible contribution from the slowly renaturing unique sequences.

Fig. 1 gives the length distribution (determined on electron micrographs) of the single stranded fragments obtained by heat denaturation of a typical DNA preparation. Since this material will reassociate at a much faster rate than the sonic fragments used in the previous studies [1, 2], suitable annealing conditions (0.3 M NaCl; 53°C) were chosen so as to slow the reassociation, and hence make the choice of optimal C_0t value less critical. The arrow in Fig 2. indicates the chosen criterion. Total *P. berghei* DNA denatured and renatured to this C_0t value was diluted 100 times with cold TE buffer to stop nucleation, but not zippering of nucleated duplexes, and then mounted

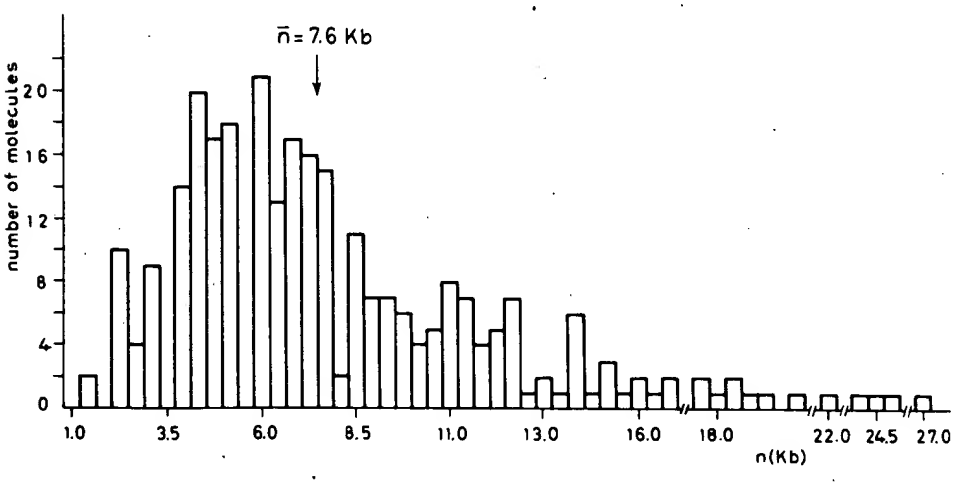


Fig. 1. Length distribution of total heat denatured *P. berghei* DNA. The same preparation, after partial reassociation to the C_0t value indicated in Fig. 2 was used for the electron microscopic observation of reassociated structures (Fig. 3). Length measurements taken on enlarged micrographs were expressed in kilobases using the linear density value (3.85 kb μm^{-1} determined for single strands in parallel measurements on φX174 viral DNA. $\bar{n} = 7.6$ kb is the average value (first moment of the distribution).

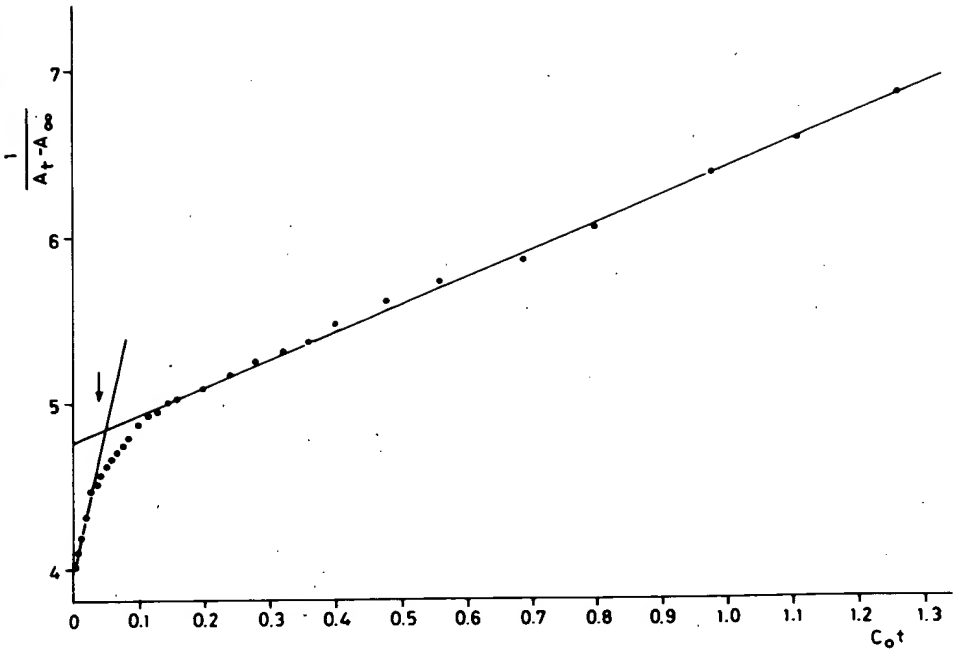


Fig. 2. Reciprocal second order plot of reassociation kinetics for *P. berghei* NK 65 DNA (infective stage), in 0.3 M NaCl, at 53°C. The biphasic curve is clearly indicative of a repetitive DNA component. The arrow indicates the C_0t value (expressed in mol s^{-1} l^{-1}) at which partial renaturation was stopped in samples observed by electron microscopy; it corresponds to advanced reassociation of the rapidly renaturing repetitive component, with a negligible contribution from slowly renaturing unique DNA to renatured structures.

for electron microscopy in the presence of formamide. Together with filaments which are too tangled to be interpreted, one can easily identify in electron micrographs several types of reassociated structures, typical examples of which are shown in Fig. 3. Intrastrand reassociation leads to the appearance of snapbacks or of loops formed predominantly by inverted repeats (loops possessing a stem) while direct repeats appear very seldom or are too short to be clearly identified; interstrand reassociation is clearly revealed as H shaped structures, possessing 4 free extremities. Quantitative measurements and statistical evaluation of frequency and length distribution of reassociated structures are not practicable, essentially for two reasons. Firstly the need to neglect uninterpretable tangled structures introduces a bias in the statistical sample and, secondly, the quality of the electron micrographs does not allow a clearcut distinction between single and double strands. Consequently there is no way of deciding whether linear (two ended) structures contain duplex regions.

On the limited sample of 3- and 4-ended molecules, where single stranded tails can be easily identified, their average length was determined. This estimate indicates that there is one reassociated (repetitive) structure per 8000 base pairs of unique DNA.

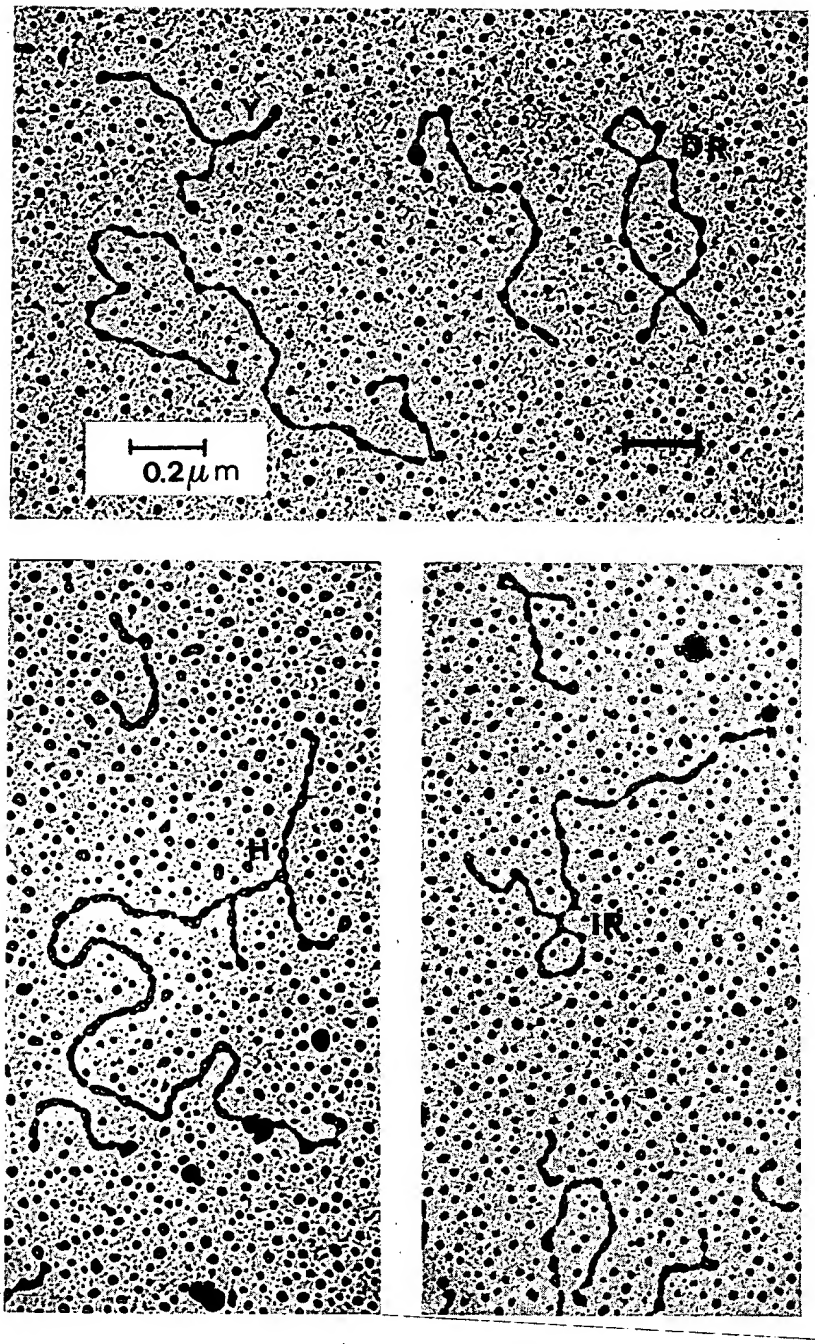


Fig. 3. Examples of rapidly reassociating repetitive structures observed in total *P. berghei* NK 65 DNA denatured and renatured to suitable C_0t value (indicated by arrow in Fig. 2). Y, snapback; IR, inverted repeat; DR, direct repeat; H, interstrand duplex.

When taking into account the initial length distribution (Fig. 1) the implication is that repetitive sequences (reassociated at low C_0t value) are widely dispersed and do not occupy preferential positions along the genome.

Presence of circular DNA molecules. In the course of the preceding [1, 2] and of the present work, total DNA preparations were often and extensively screened by electron microscopy for the presence of circular DNA molecules, and in particular those corresponding to mitochondrial DNA. Consistently negative results indicated that the usual extraction procedure was probably too drastic to preserve circularity. In order to reveal the presence of circular forms in enriched preparations, we adopted a simplified version of the method described by Kilejian [7] for the avian malarial agent *P. lophurae* (see Materials and Methods). Both a 'nuclear' fraction, formed by the pellets of the low speed centrifugations, and a 'mitochondrial' fraction (generated by the 10000 rpm centrifugation) were subjected to the extraction treatments, which do not introduce a DNAase treatment. In these conditions, the 'mitochondrial' fraction was expected to contain also nuclear DNA fragments, not sedimented with whole nuclei, while the 'nuclear' fraction should contain little or no mitochondrial DNA (some mitochondria can however be present in the pellets of the first, low speed centrifugations).

In effect, electron microscopic observation of the 'mitochondrial' DNA preparation revealed, among many linear fragments, several circular molecules of similar contour length (11.3 ± 0.4 μm). Examples are shown in Fig. 4. Only one similar form could be found in extensive examination of the 'nuclear' preparation. A few smaller circles (2–9 μm long) were present in both preparations.

Parallel extractions of rat lymphocytes showed, under the same conditions, circular forms of about 5 μm contour length, which correspond to mammalian mitochondrial DNA.

The length we determined for plasmodial mitochondrial DNA may be slightly altered by residual ethidium bromide intercalation persisting after dialysis [8]. A 7% correction, as indicated by Freifelder for this effect [8] would bring this value from 11.3 to 10.5 μm, which is in perfect agreement with the length (10.3 ± 0.2 μm) determined for mtDNA from *P. lophurae* [7].

The simplified extraction method we followed proved apt to preserve the integrity of mtDNA molecules, although it does not allow their isolation. A rough estimate of their relative abundance may be given as about 0.1% of the total DNA; an order of magnitude less than for the other circular forms.

Purification of the repetitive fraction by hydroxyapatite chromatography. Purification of the rapidly renaturing component from total DNA is essential not only to obtain a better characterization of the repetition length, but also to obtain a suitable probe to be used in hybridization studies of its distribution in the genome (paper in preparation).

ıl *P. berghei* NK 65 DNA
Y, snapback; IR, inverted

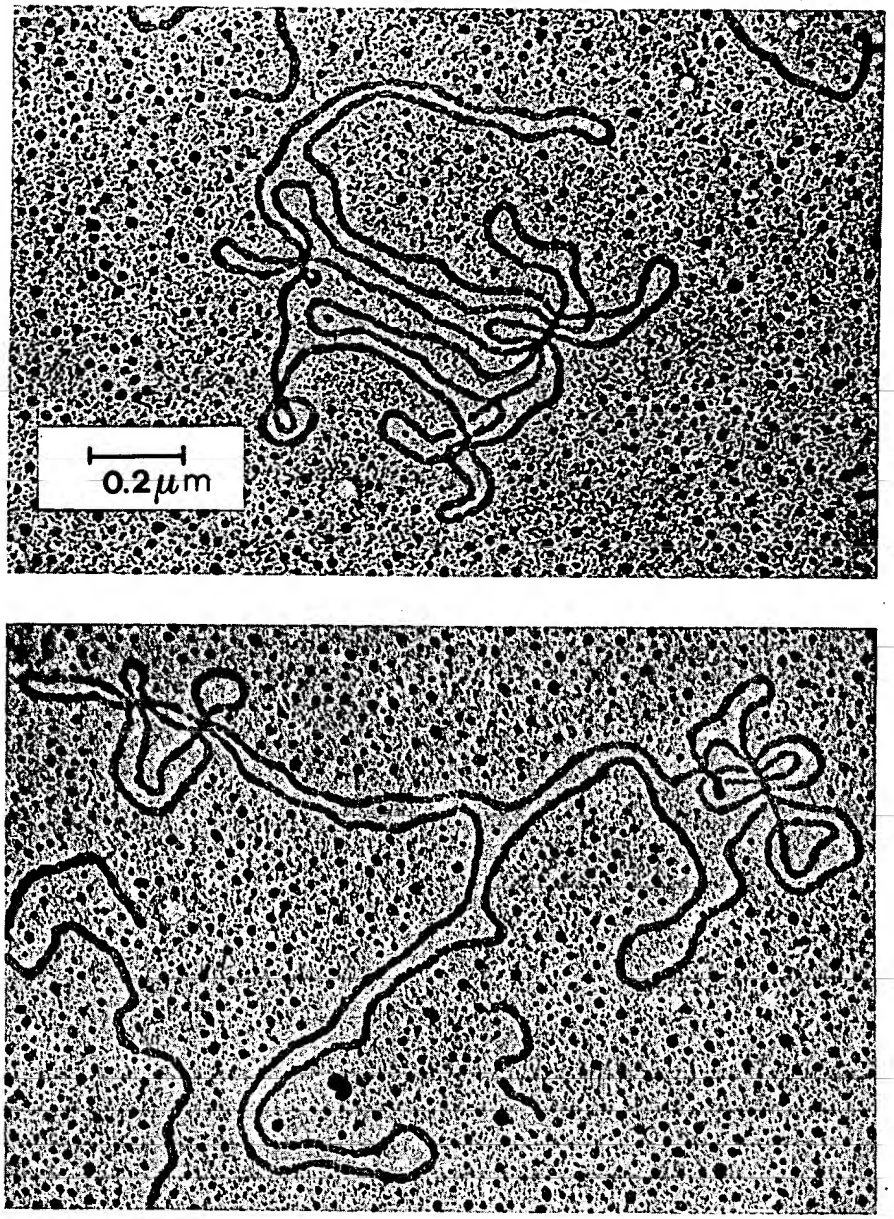


Fig. 4. Examples of circularly closed DNA molecules with contour length of about 11 μm observed in preparations enriched in mitochondrial DNA.

DNA purified by the usual extraction procedure [1, 2], but finally dialysed against 0.36 M PB was fragmented by sonication, denatured and reannealed to a C_0t value satisfying the condition already discussed (almost complete renaturation of the rapid-

ly reassociating component with negligible contribution of the unique fraction to the reassociated material). Spectrophotometric kinetic measurements performed in this particular solvent (0.36 M PB) indicated that the chosen C_0t value corresponded to 13% reassociation of the initially unpaired nucleotides.

Immediately after reaching the chosen value, the partially renatured DNA solution was diluted and loaded onto HAP columns. Parallel trials with native and denatured DNA (Fig. 5a and b) demonstrate the retention of double stranded material on the column while the PB concentration is kept at 0.12 M, and its elution by 0.4 M PB. For these experiments the recovery was between 90% and 100%, both for the double and single stranded material.

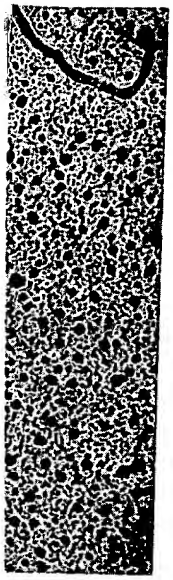
Fig. 5c shows the elution pattern obtained for the partially renatured DNA. When considering that retained material is most probably composed of duplexes with single stranded tails, it is not surprising to find that it amounts to 18% of the total eluted DNA, a percentage higher than that estimated from spectrophotometric data (see also the discussion of this effect in ref. 2). We did not introduce treatments with single strand specific nucleases because we were also interested in the environment of the repetitive sequences. Column fractions were pooled as indicated in Fig. 5c. Aliquots of pools A and B were tested for renaturing kinetics in order to verify how efficient the separation of the fast- from the slow-renaturing material had been. Curves displayed in Fig. 6 show that the material retained by the column (B) follows a pure second order kinetic, while the material eluted at low buffer molarity (A) contains a major slow-renaturing component, but also a small fast-renaturing fraction. This is probably due to repetitive sequences too short, in comparison with their single stranded tails, to be retained by the column.

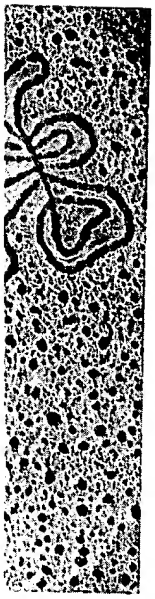
The degree of separation which can be obtained is influenced by the average size of the sonic fragments. In our hands at least, attempts by this method to fractionate DNA fragments greater than 10^3 base pairs yielded a mixture of repetitive and unique DNA (as judged from the biphasic renaturation curves) both in A and B.

Material eluted at 0.4 M PB from several columns was pooled; a small aliquot used for characterization by electron microscopy (see following paragraph) while the major part was precipitated in ethanol and stored at −20°C, for subsequent hybridization studies.

Electron microscopic characterization of the repetitive fraction purified by HAP chromatography. Fig. 7 shows some typical aspects observed by electron microscopic examination of the repetitive fraction (B) purified by hydroxyapatite chromatography. Unlike the case of partially renatured total DNA (Fig. 3) linear fragments here can be assumed to be double stranded, since they are retained by the column.

However it is impossible to tell from electron micrographs whether these linear duplexes contain some single stranded portions. The same inability to distinguish single from double stranded filaments prevents us from deciding which arm in a Y-shaped structure is actually a duplex (or a snapback). Only H forms do not present





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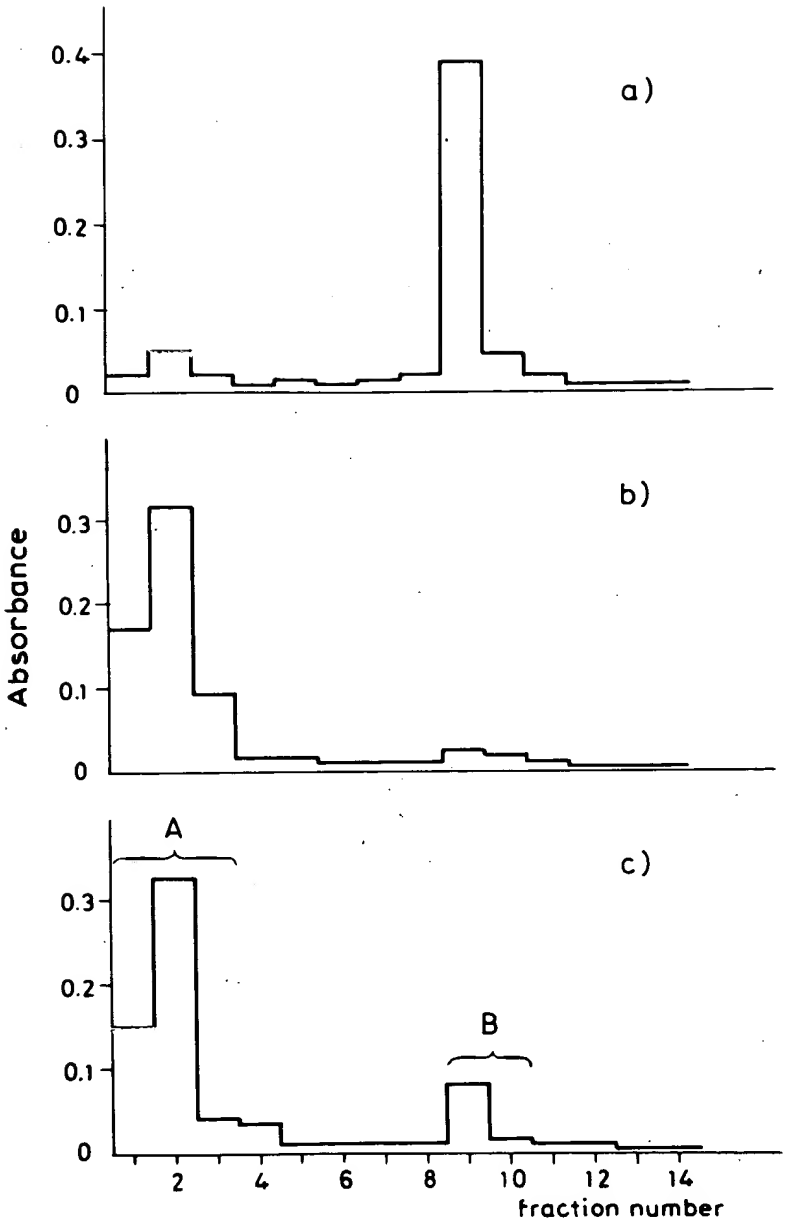

Fig. 5. The performance of hydroxyapatite columns is illustrated in (a) for native and in (b) for heat denatured DNA. Phosphate buffer concentration was 0.12 M during the elution of the first 8 fractions and was increased in a single step to 0.4 M from fraction 9 onwards. (c) gives the elution pattern of *P. berghei* DNA renatured to a C_0t value satisfying the criterion of almost complete reassociation of the fast component and negligible renaturation of unique DNA. This latter is eluted from the column as denatured material (A), while fast reassociating, double stranded fragments are recovered at higher buffer molarity (B).

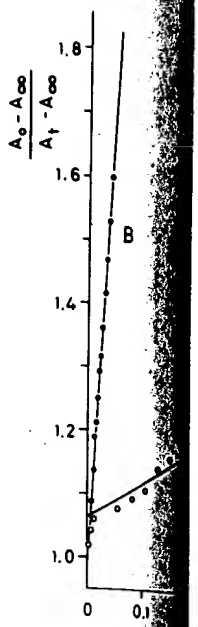

Fig. 6. Reciprocal sec
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DISCUSSION

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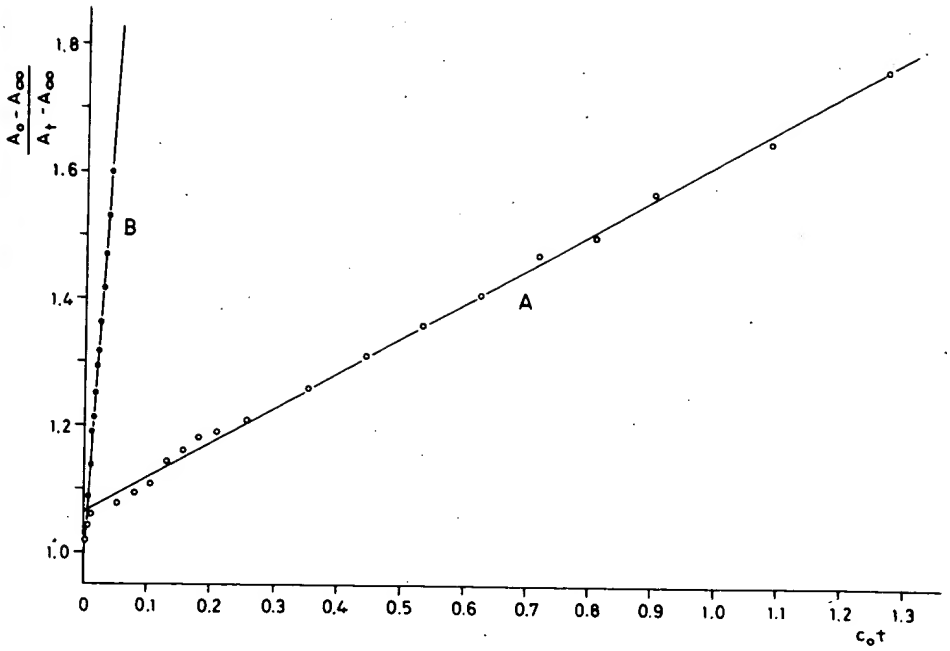

Fig. 6. Reciprocal second order plots of the renaturation kinetics followed by fractions A and B (see Fig. 5). Fraction B (●) exhibits a high renaturation rate, and follows pure second order kinetics, as expected for the purified repetitive fraction. Fraction A (○) has a predominant slow renaturing (unique) component, but still contains some fast renaturing material.

ambiguities. Certain criteria therefore had to be chosen in order to extract quantitative information from these images: we considered linear filaments as complete duplexes, while for the Y-shaped structures the average length of the three arms was taken. Fig. 8 presents the length distribution of reassociated structures obtained under these somewhat arbitrary assumptions; the length distribution of the central bar of H-shaped structures also reported in Fig. 8 (dashed line, scale at right), although poorly populated, confirms their validity.

About 90% of the measured structures fall in classes of length between 400 and 1400 base pairs, with an average length of 800 bp. As far as interspersion is concerned, no quantitative data can be obtained from the observation of the purified repetitive fraction, since DNA had been fragmented before loading the column. Nevertheless, the relatively high proportion of single stranded tails present in reassociated structures provides an indication in favour of it being highly interspersed with unique DNA portions.

DISCUSSION

Evidence obtained from electron microscopic analysis of total, partially renatured

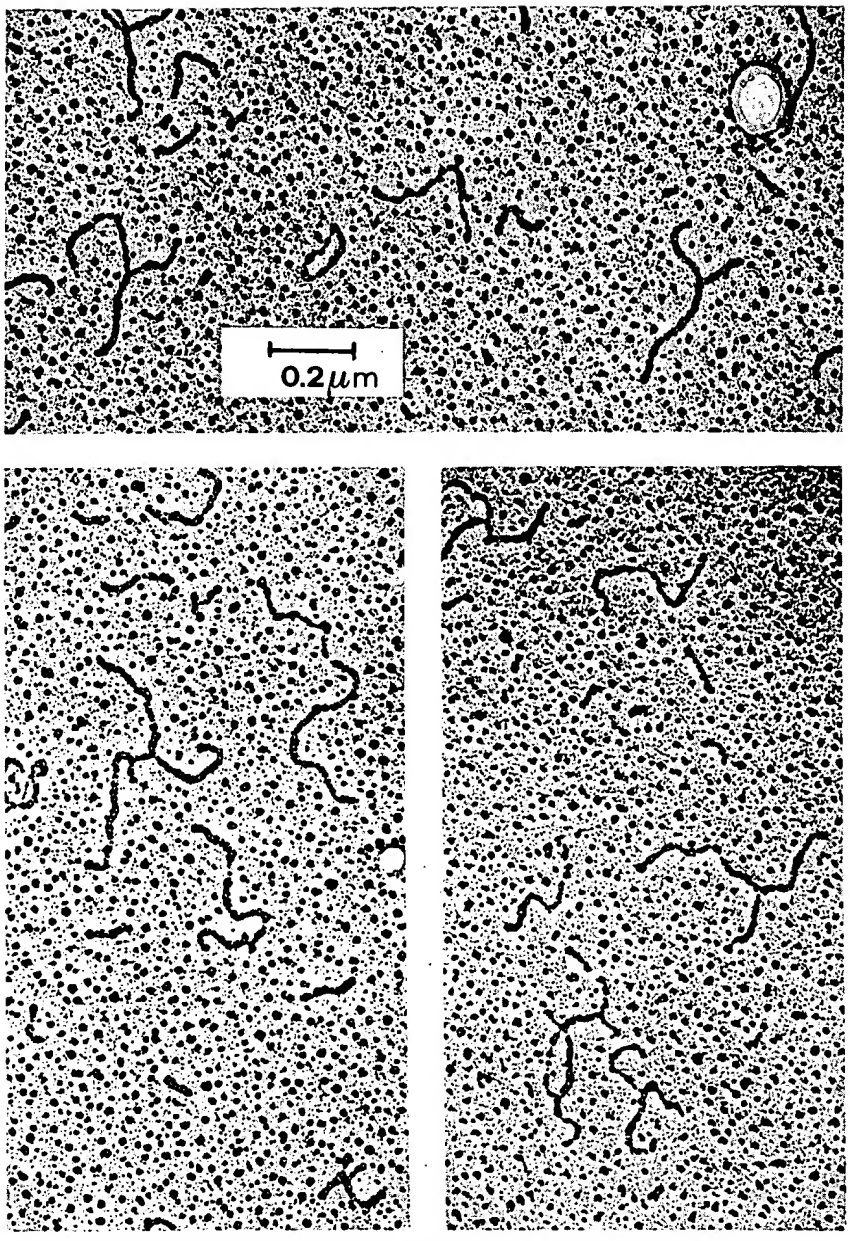

Fig. 7. Typical aspects observed in HAP purified repetitive DNA (fraction B in Fig. 5).

DNA and of its purified repetitive fraction indicated that *P. berghei* genome contains repetitive sequences several hundred base pairs long (400–1400 bp) largely interspersed with unique DNA (one in approx. 8000 bp of unique DNA).

This conclusion is in contrast with what was stated in a previous paper [1], on the

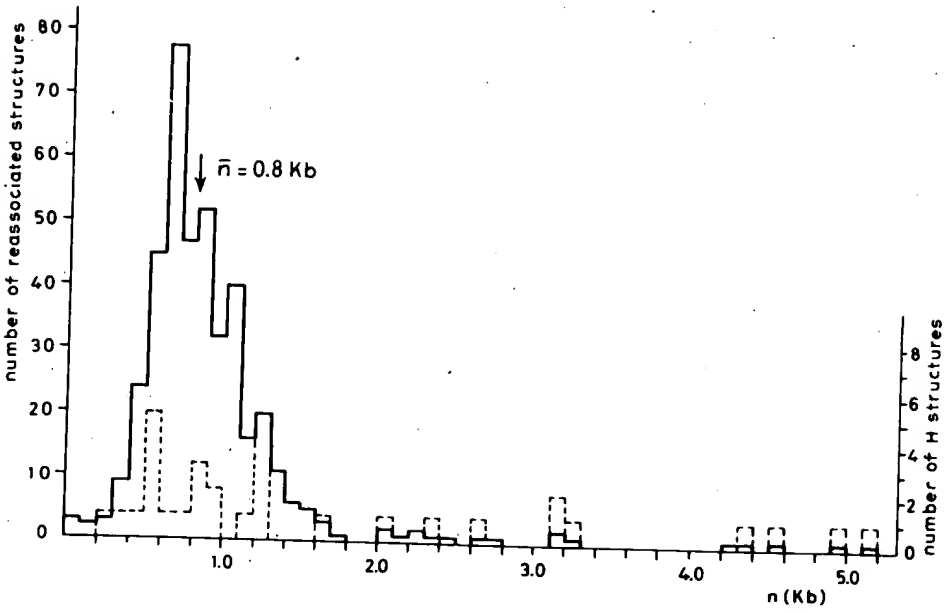

Fig. 8. Length distribution of duplex regions observed in purified repetitive DNA (fraction B, Fig. 5) as linear, Y-shaped or H-shaped structures. The criteria followed in the case of linear and of Y-shaped structures (see text) are confirmed by comparison with the distribution of the unambiguous duplex regions (central bar) in H-shaped structures (scale at right; dashed histogram). Measurements taken on enlarged micrographs were expressed in kbases using the linear density value of 3.25 kb μm^{-1} determined in parallel measurements on PRB 322.

basis of an incorrect analysis of reassociation kinetics. As clarified by Goltsov et al. [9], classical analysis [3] fails when applied to relatively short repetitive sequences interspersed with unique DNA and yields grossly overestimated values of the repetition length [10]. Data described in the present work confirm this point of view.

In the probably oversimplified hypothesis that the repetitive genome fraction (approx. 2×10^9 daltons $= 3 \times 10^6$ bp) consists of a single family with repetition length equal to the average value of our distribution (800 bp), then the reiteration frequency would be of the order of 4×10^3. This estimate is subject to change according to the number of repetitive families present, or to possible non-homogeneous distribution of repetitive DNA among the different intraerythrocytic forms. It is in effect conceivable that gametocytes contain a higher proportion of repetitious DNA, and in this case repetition frequency would be greater.

The presence in *P. berghei* of mitochondrial DNA of the same length as that purified from *P. lophurae* [7] has been ascertained. Its relative abundance is so low that its purification encounters practical limits in terms of the amount of infected blood required (about 10 times higher than the amounts thus far used). On the other hand, this low value is in itself an indication that repetitive DNA, amounting to 16–18% of the whole genome, cannot be essentially of mitochondrial location.

ACKNOWLEDGEMENT

Thanks are due to the Department of Ultrastructures and to the Department of Parasitology for the continuous and reliable technical assistance which made this work possible.

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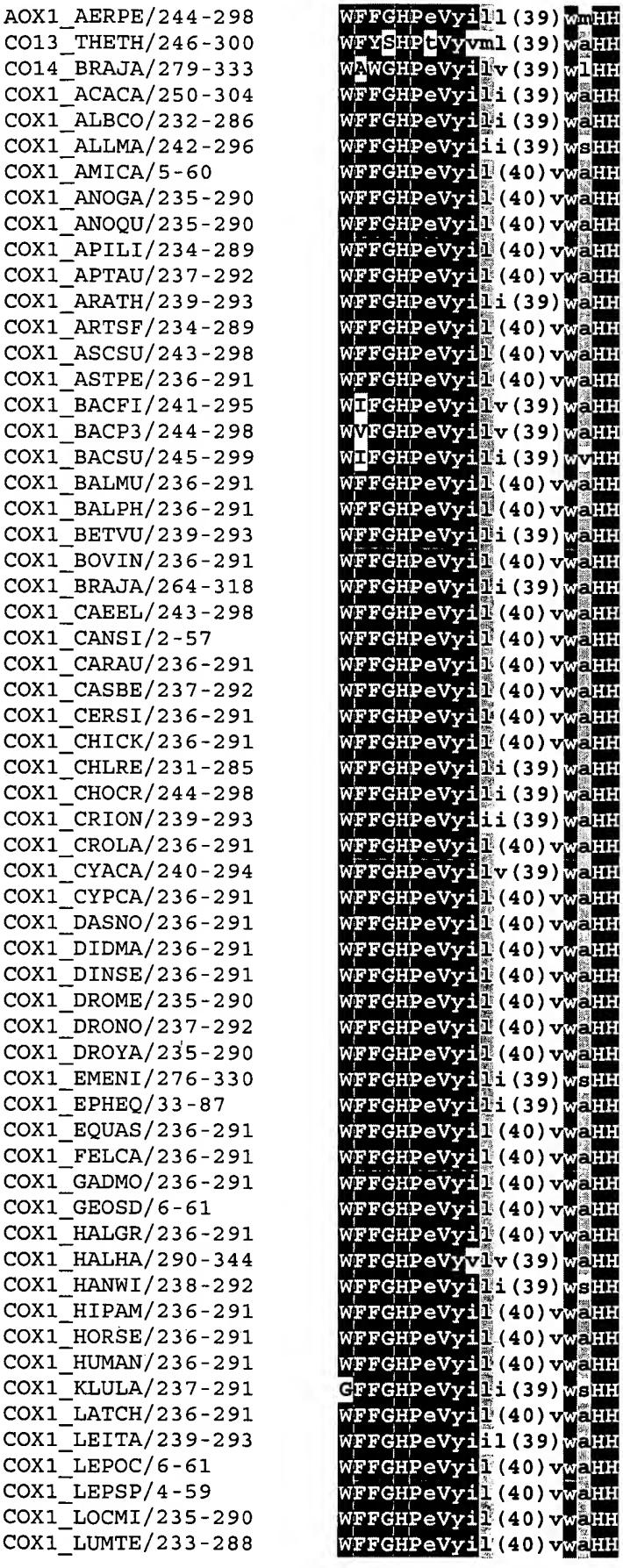
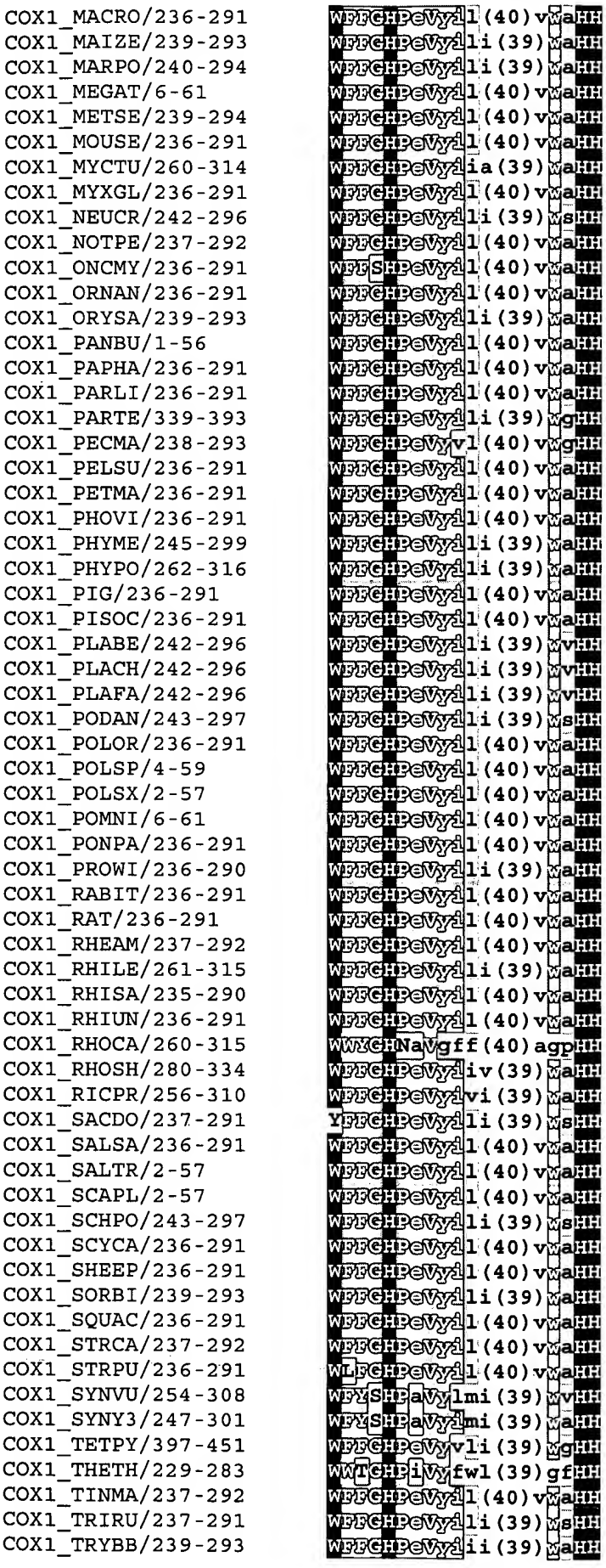
5,000 P. Berghei sporozoites. Ten days after injection the animals were bled in the presence of heparin. The blood was spun down and the buffy coat removed. The pellet was washed, resuspended and passed through a glass wool column. The purified red cells were treated with 0.015% (w/b) Saponin (Sigma, St. Louis, MO) and incubated at 37.degree. C. for 15 minutes. The released parasites were washed with PBS, homogenized in the presence of 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM EDTA and 0.5 % SDS. After treatment with proteinase K, this material was extracted twice with a chloroform-isoamyl alcohol-phenol mixture as above and precipitated with ethanol. This was followed with a second cycle of proteinase K-RNAase treatment, organic solvent extraction and ethanol precipitation. The pellet was dissolved in the water and the DNA concentration measured. Serial dilutions of DNA were immobilized onto nitrocellulose filters and hybridized with a probe. Preparation of the probe is described below.

Detailed Description Text (45):
As can be seen in FIG. 4, a linear relationship existed between the amount of DNA bound to the filter and the amount of radioactivity retained after hybridization. This linear relationship existed between 100 pg to one microgram of P. berghei DNA immobilized on nitrocellulose filters. The open circles and the closed circles in FIG. 4 represent the results of two different experiments. Each value represents the mean of three filters. Standard deviations were negligible.

Detailed Description Text (50):
The DNA probe (p-263-1) used to assay sporozoite infectivity was prepared as follows: A 2.2 Kb sequence of P. berghei origin was obtained from a pBR322/BamH1 library containing Sau3A fragments of P. berghei total DNA. This element is present in at least 100 copies in the genoma of P. berghei as determined by standard dot blot hybridization. Total P. berghei sporozoite DNA was digested with Sau3A and fractionated on a 1% low melting point agarose (IBI, New Haven, CT) gel. The 2.2 kb region of the gel was excised and the DNA recovered by melting the agarose and extracting the DNA according to the manufacturers instructions.

CLUSTAL format alignment of matches to PROSITE PS00077

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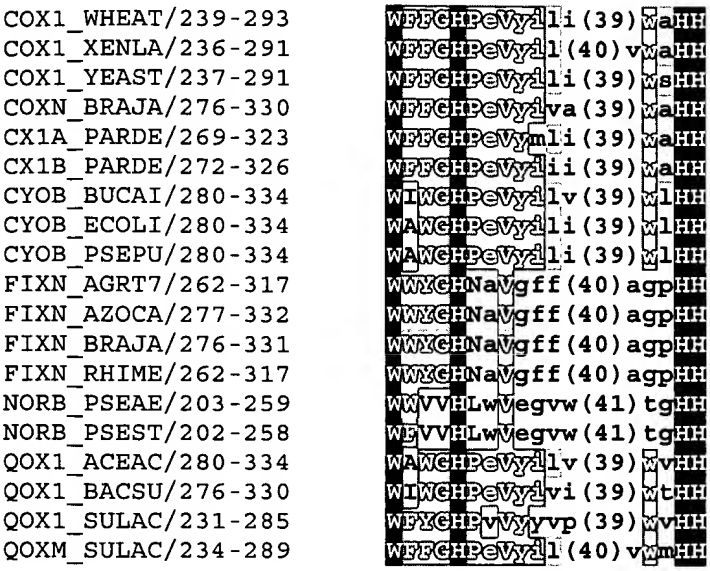
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NiceSite View of PROSITE: PS00077

General information about the entry	
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Accession number	PS00077
Entry type	PATTERN
Date	APR-1990 (CREATED); NOV-1997 (DATA UPDATE); JUL-1998 (INFO UPDATE).
PROSITE documentation	PDOC00074
Name and characterization of the entry	
Description	Heme-copper oxidase catalytic subunit, copper B binding region signature.
Pattern	[YWG]-[LIVFYWTA](2)-[VGS]-H-[LNP]-x-V-x(44,47)-H-H.
Numerical results	

SWISS-PROT release number: **40.7**, total number of sequence entries in that release: **103373.**

- Total number of hits in SWISS-PROT: **141 hits in 141 different sequences**
- Number of hits on proteins that are known to belong to the set under consideration: **141 hits in 141 different sequences**

Number of hits on proteins that could potentially belong to the set under consideration: **0 hits in 0 different sequences**

- Number of false hits (on unrelated proteins): **0 hits in 0 different sequences**

Number of known missed hits: **4**
Number of partial sequences which belong to the set under consideration, but which are not hit by the pattern or profile because they are partial (fragment) sequences: **10**
Precision (true hits / (true hits + false positives)): **100.00 %**
Recall (true hits / (true hits + false negatives)): **97.24 %**

Comments

Taxonomic range: **Archaebacteria, Eukaryotes, Prokaryotes (Bacteria)**
Maximum known number of repetitions of the pattern in a single protein: **1**
`Interesting' site in the pattern: **4,copper**
`Interesting' site in the pattern: **9,copper**
`Interesting' site in the pattern: **10,copper**

Cross-references

True positive hits:

AOX1_AERPE (Q9YDX6), CO13_THETH (P98005), CO14_BRAJA (P98057),
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COX1_AMICA (P29643), COX1_ANOGA (P34838), COX1_ANOQU (P33504),
COX1_APILI (P20374), COX1_APTAU (O03515), COX1_ARATH (Q07063),
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SWISS-PROT	COX1_RHOSH (P33517), COX1_RICPR (O54069), COX1_SACDO (P98001), COX1_SALSA (Q9ZZM6), COX1_SALTR (P29653), COX1_SCAPL (P29654), COX1_SCHPO (P07657), COX1_SCYCA (O79403), COX1_SHEEP (O78749), COX1_SORBI (P05502), COX1_SQUAC (Q9ZZ52), COX1_STRCA (O21399), COX1_STRPU (P15544), COX1_SYNVU (P50676), COX1_SYNY3 (Q06473), COX1_TETPY (P11947), COX1_THETH (Q56408), COX1_TINMA (O03554), COX1_TRIRU (Q01555), COX1_TRYBB (P04371), COX1_WHEAT (P08741), COX1_XENLA (P00398), COX1_YEAST (P00401), COXN_BRAJA (P98000), CX1A_PARDE (P08305), CX1B_PARDE (P98002), CYOB_BUCAI (P57543), CYOB_ECOLI (P18401), CYOB_PSEPU (Q9WWR2), FIXN_AGRT7 (P98055), FIXN_AZOCA (P98056), FIXN_BRAJA (Q03073), FIXN_RHIME (Q05572), NORB_PSEAE (Q59647), NORB_PSEST (P98008), QOX1_ACEAC (P98009), QOX1_BACSU (P34956), QOX1_SULAC (P98004), QOXM_SULAC (P39481) **False negative hits (sequences which belong to the set under consideration, but which have not been picked up by the pattern or profile):** COX1_CANFA (Q9ZZ64), COX1_OENBE (P08743), COX1_PEA (P12786), COX1_SOYBN (P07506) **`Potential' hits (partial sequences which belong to the set under consideration, but which are not hit by the pattern or profile because they are partial (fragment) sequences):** COX1_ALBTU (Q09333), COX1_ANAPL (P50656), COX1_CAPHI (Q36347), COX1_CHOBI (P50668), COX1_CHOFU (P50669), COX1_CHOOC (P50670), COX1_CHORO (P50671), COX1_COTJA (P24984), COX1_GOMVA (P29646), COX1_MYTED (P41774) **Retrieve an alignment of SWISS-PROT true positive hits:** [Clustal format, color, condensed view] [Clustal format, color] [Clustal format, plain text] [Fasta format]
PDB new! [Detailed view]	1OCC; 1OCO; 1OCR; 1OCZ; 2OCC; 1EHK; 1AR1; 1FFT;

View entry in original PROSITE format
View entry in raw text format (no links)
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If you would like to retrieve all the SWISS-PROT entries referenced in the

DR lines of this entry (with the exception of false positive hits) , you can enter a file name. These entries will then be saved to a file under this name in the directory `outgoing` of the ExPASy anonymous ftp server, from where you can download it. (Please note that this temporary file will only be kept for 1 week.)

File name:

Reset or Create file

NiceProt View of TrEMBL: Q26218

Printer-friendly view | Quick BlastP search

[General] [Name and origin] [References] [Comments] [Cross-references] [Keywords] [Features] [Sequence] [Tools]

General information about the entry	
Entry name	**Q26218**
Primary accession number	**Q26218**
Secondary accession numbers	None
Entered in TrEMBL in	Release 01, November 1996
Sequence was last modified in	Release 08, November 1998
Annotations were last modified in	Release 23, February 2003
Name and origin of the protein	
Protein name	**Cytochrome c oxidase subunit 1 [Fragment]**
Synonyms	**EC 1.9.3.1** **Cytochrome c oxidase polypeptide I**
Gene name	***COX1***
From	Plasmodium yoelii [TaxID: 5861]
Taxonomy	Eukaryota; Alveolata; Apicomplexa; Haemosporida; Plasmodium.

References

[1] SEQUENCE FROM NUCLEIC ACID.
STRAIN=17X;
MEDLINE=89364999; PubMed=2549417; [NCBI, ExPASy, EBI, Israel, Japan]
Vaidya A.B., Akella R., Suplick K.;
"Sequences similar to genes for two mitochondrial proteins and portions of ribosomal RNA in tandemly arrayed 6-kilobase-pair DNA of a malarial parasite.";
Mol. Biochem. Parasitol. 35:97-107(1989).

[2] SEQUENCE FROM NUCLEIC ACID.
STRAIN=17X;
MEDLINE=91061745; PubMed=1701017; [NCBI, ExPASy, EBI, Israel, Japan]
Suplick K., Morrisey J., Vaidya A.B.;
"Complex transcription from the extrachromosomal DNA encoding mitochondrial functions of Plasmodium yoelii.";
Mol. Cell. Biol. 10:6381-6388(1990).

[3] SEQUENCE FROM NUCLEIC ACID.
STRAIN=17X;
Vaidya A.B.;
Submitted (JUL-1998) to the EMBL/GenBank/DDBJ databases.

Comments

FUNCTION: Cytochrome c oxidase is the component of the respiratory chain that catalyzes the reduction of oxygen to water. Subunits 1-3 form the functional core of the enzyme complex. CO I is the catalytic subunit of the enzyme. Electrons originating in cytochrome c are transferred via the copper A center of subunit 2 and heme a of subunit 1 to the bimetallic center formed by heme a3 and copper B *(By similarity)*.
CATALYTIC ACTIVITY: 4 ferrocytochrome c + O_2 = 4 ferricytochrome c + 2 H_2O.
PATHWAY: Respiratory chain; terminal step.
SUBCELLULAR LOCATION: Integral membrane protein. Mitochondrial inner membrane *(By similarity)*.

SIMILARITY: BELONGS TO THE HEME-COPPER RESPIRATORY OXIDASE FAMILY.

Cross-references

EMBL	M29000; AAC25923.1; [EMBL / GenBank / DDBJ] -. [CoDingSequence]
HSSP	P18401; 1FFT. [HSSP ENTRY / PDB]
InterPro	IPR000883; COX1. Graphical view of domain structure.
Pfam	PF00115; COX1; 1.
PRINTS	PR01165; CYCOXIDASEI.
PROSITE	PS00077; COX1; 1.
ProtoMap	Q26218.
PRESAGE	Q26218.
ModBase	Q26218.
SWISS-2DPAGE	Get region on 2D PAGE.

Keywords

Copper; **Electron transport**; **Heme**; **Inner membrane**; **Membrane**; **Oxidoreductase**; **Respiratory chain**; **Transmembrane**; **Transport**; **Mitochondrion**.

Features

Key	From	To	Length	Description
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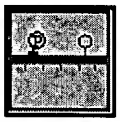 Feature table viewer

Sequence information

Length: **477 AA** [This is the length of the partial sequence]	Molecular weight: **52817 Da** [This is the MW of the partial sequence]	CRC64: **EFBD0875A8BF00D0** [This is a checksum on the sequence]

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Q26218 in FASTA format

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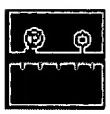
Request for annotation of this TrEMBL entry

Direct BLAST submission at EMBnet-CH/SIB (Switzerland)

Direct BLAST submission at NCBI (Bethesda, USA)

ScanProsite, MotifScan

Sequence analysis tools: ProtParam, ProtScale, Compute pI/Mw, PeptideMass, PeptideCutter, Dotlet (Java)



Feature table viewer (Java)

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SV   M29000.1
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DT   04-MAR-2000 (Rel. 63, Last updated, Version 7)
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DE   and cytochrome b (cob) gene, complete cds.
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OC   Eukaryota; Alveolata; Apicomplexa; Haemosporida; Plasmodium.
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RA   Vaidya A.B., Akella R., Suplick K.;
RT   "Sequences similar to genes for two mitochondrial proteins and portions of
RT   ribosomal RNA in tandemly arrayed 6-kilobase-pair DNA of a malarial
RT   parasite";
RL   Mol. Biochem. Parasitol. 35(2):97-107(1989).
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RA   Suplick K., Morrisey J., Vaidya A.B.;
RT   "Complex transcription from the extrachromosomal DNA encoding mitochondrial
RT   functions of Plasmodium yoelii";
RL   Mol. Cell. Biol. 10(12):6381-6388(1990).
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RT   ;
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RT   ;
RL   Submitted (09-JUL-1998) to the EMBL/GenBank/DDBJ databases.
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1: Exp Parasitol 1993 Sep;77(2):136-46 Related Articl

ELSEVIER SCIENCE FULL-TEXT ARTICLE

Plasmodium berghei: partial purification and characterization of the mitochondrial cytochrc oxidase.

Krungkrai J, Krungkrai SR, Bhumiratana A.

Department of Biochemistry, Faculty of Medicine, Chulc University, Bangkok, Thailand.

Mitochondria from a rodent malarial parasite (Plasmodiu berghei) were successfully purified by differential centrifugation and 22% Percoll density gradient separa purified mitochondria from the erythrocytic stages of parasite had a density of 1.05 and were found to be heterogeneous by transmission electron microscopy and rhodamine 123 fluorescence microscopy. Three marker dihydroorotate dehydrogenase, cytochrome c reductase cytochrome c oxidase, were assessed during the organe separation. Purification of cytochrome c oxidase was ca from the purified mitochondria by using combination te of detergent solubilization and reduced cytochrome c-a affinity chromatography. The 560-fold purified enzyme 3.6% yield was obtained and it had low catalytic efficie a kcat/Km of 5.9 x 10(-5) M-1 x min-1. The native form

a kcat/Km of 5.9 x 10(-5) M-1 x min-1. The native form
enzyme, determined by a gel filtration column on fast pr
liquid chromatography, was found to be an oligomeric st
with a minimal molecular weight of 670 kDa. The malaric
enzyme was analyzed by sodium dodecyl sulfate-polyacr
gel electrophoresis and then compared to the enzyme o
from host liver cells. These results suggested that the
purified enzyme from the parasite was not different fr
host mammalian cells. The importance of the enzyme in
erythrocytic phase of the parasite is discussed as a par
simple electron transport system in mitochondrion linke
limited oxygen utilization and pyrimidine de novo biosyn

PMID: 8397100 [PubMed - indexed for MEDLINE]

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DATE: Wednesday, December 11, 2002

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L2	lsu.clm.	10	L2
L3	(large.clm. same (subunit or sub-unit)).clm.	62	L3
L4	L3 and l1	4	L4
L5	L3 and (malar$ or plasmod$ or berghei or falciparum).clm.	2	L5
L6	(malar$ or plasmod$ or berghei or falciparum).clm.	593	L6
L7	L6 and (method$ or process$).clm.	492	L7
L8	(dna or cdna or antisense or anti-sense or rrna or r-rna or nucleic or nucleotide or polynucleotide or nuclear or chromosomal or plasmid or plastid or plastidlike or plastid-like or probe or primer).clm.	66388	L8
L9	L8 and l6	104	L9
L10	L9 and l7	78	L10
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L12	L11 same (dna or cdna or antisense or anti-sense or rrna or r-rna or nucleic or nucleotide or polynucleotide or nuclear or chromosomal or plasmid or plastid or plastidlike or plastid-like or probe or primer)	33	L12
L13	('4707445')[PN]	1	L13

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☐ 1. Document ID: US 6221355 B1

L5: Entry 1 of 2 File: USPT Apr 24, 2001

DOCUMENT-IDENTIFIER: US 6221355 B1
TITLE: Anti-pathogen system and methods of use thereof

CLAIMS:

11. The anti-pathogen system of claim 1, wherein the pathogen-specific protease cleavage sites are selected from the group consisting of cytomegalovirus (CMV), herpes simplex virus type-1 (HSV-1); hepatitis virus type C (HCV); P. falciparum, HIV-1, HIV-2 and Kaposi's sarcoma-associated herpes virus (KSHV) protease cleavage sites.

12. The anti-pathogen system of claim 1, wherein the fusion protein comprises covalently linked in sequence: 1) TAT or a protein transducing fragment thereof, 2) a caspase-3 prodomain, 3) a first HSV-1 protease cleavage site, 4) a caspase-3 large subunit, 5) a second HSV-1 protease cleavage site, and 5) a caspase-3 small subunit, wherein the large and small subunits dimerize to form a cytotoxin.

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw. Desc
Image

☐ 2. Document ID: US 5766597 A

L5: Entry 2 of 2 File: USPT Jun 16, 1998

DOCUMENT-IDENTIFIER: US 5766597 A
TITLE: Malaria recombinant poxviruses

CLAIMS:

1. A recombinant poxvirus containing therein DNA from Plasmodium falciparum coding for at least one Plasmodium antigen in a nonessential region of the poxvirus genome wherein the poxvirus expresses the at least one antigen and the poxvirus is selected from the group consisting of:

(i) recombinant vaccinia virus wherein regions C7-K1L, J2R, B13R+B14R, A56R and I4L have been deleted therefrom, or wherein the open reading frames for the thymidine kinase gene, the hemorrhagic region, the A type inclusion body region, the hemagglutinin gene, the host range gene region, and the large subunit, ribonucleotide reductase have been deleted therefrom;

(ii) NYVAC vaccinia virus; and

(iii) ALVAC canarypox virus.

2. A recombinant poxvirus as in claim 1 wherein said DNA codes for a Plasmodium antigen from each of sporozoite, liver, blood and sexual stages of the Plasmodium life cycle.

3. A recombinant poxvirus as in claim 1 wherein said Plasmodium antigen is selected from the group consisting of SERA, ABRA, Pfhsp70, AMA-1, Pfs25, Pfs16, CSP, PfSSP2, LSA-1, LSA-1-repeatless, MSA-1 N-terminal p83, MSA-1 C-terminal gp42 and MSA-1 and combinations thereof.

9. A composition as in claim 8 wherein said Plasmodium antigen is selected from the group consisting of SERA, ABRA, Pfhsp70, AMA-1, Pfs25, Pfs16, CSP, PfSSP2, LSA-1, MSA-1, LSA-1-repeatless, MSA-1 N-terminal p83, MSA-1 C-terminal gp42, and combinations thereof.

11. The composition of claim 10 wherein the Plasmodium antigen is selected from the group consisting of SERA, ABRA, Pfhsp70, AMA-1, Pfs25, CSP, PfSSP2, LSA-1, MSA-1, LSA-1-repeatless, MSA-1 N-terminal p83, MSA-1 C-terminal gp42 and combinations thereof.

15. A method for producing at least one Plasmodium falciparum antigen, said method comprising infecting a cell in vitro with a recombinant poxvirus as claimed in claim 1.

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Terms	Documents
L3 and (malar$ or plasmod$ or berghei or falciparum).clm.	2

Display Format: KWIC Change Format

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L10: Entry 23 of 78 File: USPT Aug 8, 2000

DOCUMENT-IDENTIFIER: US 6100067 A
TITLE: Molecules containing at least one peptide sequence carrying one or several epitopes characteristic of a protein produced by P. falciparum at the sporozoite stage and in the hepatocytes

CLAIMS:

1. A nucleotide sequence encoding a portion of a Plasmodium falciparum protein selected from the group consisting of

(1) an amino acid sequence of between 10 and 87 consecutive amino acids of the following sequence:

Glu-Phe-Arg-Val-Ser-Thr-Ser-Asp-Thr-Pro-Gly-Gly-Asn-Glu-Ser-Ser-Ser-Ala-Se r
-Pro-Asn-Leu-Ser-Gly-Ala-Arg-Glu-Lys-Lys-Asp-Glu-Lys-Glu-Ala-Ser-Glu-Gln-Gl
y-Glu-Glu-Ser-His-Lys-Lys-Glu-Asn-Ser-Gln-Glu-Ser-Ala-Asn-Gly-Lys-Asp-Asp-V
al-Lys-Glu-Glu-Lys-Lys-Thr-Asn-Glu-Lys-Lys-Asp-Asp-Gly-Lys-Thr-Asp-Lys-Val-
Gln-Glu-Lys-Val-Leu-Glu-Lys-Ser-Pro-Lys-Glu-Phe;

(2) the 87 amino acids according to (1);

(3) the amino acid sequence:

Ala-Arg-Glu-Lys-Lys-Asp-Glu-Lys-Glu-Ala-Ser-Glu-Gln-Gly-Glu-Glu-Ser-His-Ly s
-Lys-Glu-Asn-Ser-Gln-Glu-Ser-Ala; and

(4) the amino acid sequence:

Asn-Gly-Lys-Asp-Asp-Val-Lys-Glu-Glu-Lys-Lys-Thr-Asn-Glu-Lys-Lys-Asp-Asp-Gl y
-Lys-Thr-Asp-Lys-Val-Gln-Glu Lys-Val-Leu-Glu-Lys-Ser-Pro-Lys-Glu-Phe,

wherein said amino acid sequence is recognized by antibodies recognizing the sporozoite and hepatic stages of Plasmodium falciparum and is not recognized by antibodies recognizing the blood stage of Plasmodium falciparum.

2. The nucleotide sequence according to claim 1, wherein said nucleotide sequence encodes an amino acid sequence of between 10 and 87 consecutive amino acids of the following sequence:

Glu-Phe-Arg-Val-Ser-Thr-Ser-Asp-Thr-Pro-Gly-Gly-Asn-Glu-Ser-Ser-Ser-Ala-Se r

-Pro-Asn-Leu-Ser-Gly-Ala-Arg-Glu-Lys-Lys-Asp-Glu-Lys-Glu-Ala-Ser-Glu-Gln-Gl
y-Glu-Glu-Ser-His-Lys-Lys-Glu-Asn-Ser-Gln-Glu-Ser-Ala-Asn-Gly-Lys-Asp-Asp-V
al-Lys-Glu-Glu-Lys-Lys-Thr-Asn-Glu-Lys-Lys-Asp-Asp-Gly-Lys-Thr-Asp-Lys-Val-
Gln-Glu-Lys-Val-Leu-Glu-Lys-Ser-Pro-Lys-Glu-Phe,

wherein said amino acid sequence is recognized by antibodies recognizing the sporozoite and hepatic stages of Plasmodium falciparum and is not recognized by antibodies recognizing the blood stage of Plasmodium falciparum.

3. The nucleotide sequence according to claim 1, wherein said nucleotide sequence encodes the 87 amino acids according to (1),

wherein said amino acid sequence is recognized by antibodies recognizing the sporozoite and hepatic stages of Plasmodium falciparum and is not recognized by antibodies recognizing the blood stage of Plasmodium falciparum.

4. The nucleotide sequence according to claim 1, wherein said nucleotide sequence encodes the amino acid sequence:

Ala-Arg-Glu-Lys-Lys-Asp-Glu-Lys-Glu-Ala-Ser-Glu-Gln-Gly-Glu-Glu-Ser-His-Ly s
-Lys-Glu-Asn-Ser-Gln-Glu-Ser-Ala,

wherein said amino acid sequence is recognized by antibodies recognizing the sporozoite and hepatic stages of Plasmodium falciparum and is not recognized by antibodies recognizing the blood stage of Plasmodium falciparum.

5. The nucleotide sequence according to claim 1, wherein said nucleotide sequence encodes the amino acid sequence:

Asn-Gly-Lys-Asp-Asp-Val-Lys-Glu-Glu-Lys-Lys-Thr-Asn-Glu-Lys-Lys-Asp-Asp-Gl y
-Lys-Thr-Asp-Lys-Val-Gln-Glu-Lys-Val-Leu-Glu-Lys-Ser-Pro-Lys-Glu-Phe,

wherein said amino acid sequence is recognized by antibodies recognizing the sporozoite and hepatic stages of Plasmodium falciparum and is not recognized by antibodies recognizing the blood stage of Plasmodium falciparum.

6. Recombinant DNA comprising a nucleotide sequence according to claim 1, wherein said sequence is inserted into a heterologous host vector.

7. Recombinant DNA according to claim 6, wherein said nucleotide sequence is preceded by a promoter and followed by a signal sequence coding for the termination of transcription.

8. A DNA or RNA primer comprising 30 to 261 nucleotides of the nucleotide sequence according to claim 1 or a sequence that hybridizes to the sequence according to claim 1 under conditions suitable for polymerase chain reaction (PCR).

9. A DNA or RNA primer comprising 30 to 261 nucleotides complementary to the nucleotide sequence according to claim 1 or a sequence that hybridizes to the sequence according to claim 1 under conditions suitable for polymerase chain reaction (PCR).

10. A recombinant vector comprising

a recombinant DNA sequence, wherein said sequence comprises a nucleotide sequence encoding a peptide according to claim 1; and

elements for expression of said peptide;

wherein said nucleotide sequence and elements are inserted into a heterologous host vector at a site, which is not essential for replication of the host vector.

11. The recombinant vector according to claim 10, wherein said recombinant DNA further comprises a promoter, which precedes said nucleotide sequence, and a signal sequence coding for the termination of transcription which follows said nucleotide sequence.

13. The recombinant vector according to claim 12, wherein said vector is plasmid DG671.

15. A method for the in vitro diagnosis of malaria comprising amplification of a DNA sequence as claimed in claim 1 or a sequence that hybridizes to the sequence according to claim 1 under conditions suitable for polymerase chain reaction (PCR), employing

a DNA or RNA primer comprising 30 to 261 nucleotides of the nucleotide sequence according to claim 1 or a complement thereof or a sequence that hybridizes to the nucleotide sequence according to claim 1 under conditions suitable for polymerase chain reaction (PCR).

16. The method according to claim 15, comprising

(1) contacting a biological sample with said primer under conditions permitting the formation of a hybridization complex between said primer and nucleotide sequences present in the biological sample; and

(2) detecting the formation of a hybrid.

17. A diagnostic kit for the detection of a nucleotide sequence according to claim 1 present in a biological sample, which nucleotide sequence hybridizes with a DNA sequence to form a hybrid, wherein said kit comprising

a probe comprising a DNA sequence according to claim 1;

a medium for carrying out a hybridization reaction between the nucleotide sequence to be detected and said probe; and

a reagent to detect the hybrid formed between nucleotide sequence present in said biological sample and said DNA sequence of claim 1;

wherein said DNA sequence, medium, and reagent are present in an amount sufficient to perform said detection.

18. An isolated nucleic acid sequence selected from the group consisting of:

(A) the sequence depicted in FIG. 1;

(B) a sequence that hybridizes with the sequence depicted in FIG. 1 under stringent hybridization conditions of 65.degree. C. in 2.times.SSC;

(C) a sequence, but for the degeneracy of the genetic code, encodes the same polypeptide as the polypeptide encoded by the sequence depicted in FIG. 1; and

(D) a fragment of any of sequences (A), (B), or (C),

wherein the sequence encodes a peptide that is recognized by antibodies recognizing the sporozoite and hepatic stages of Plasmodium falciparum and is not recognized by antibodies recognizing the blood stage of Plasmodium falciparum.

19. A nucleotide probe consisting of between 40 and 200 consecutive nucleotides from the sequence depicted in FIG. 1.

20. An isolated nucleic acid sequence as depicted in FIG. 1 or a fragment thereof, wherein the sequence or fragment thereof encodes a peptide that is recognized by antibodies recognizing the sporozoite and hepatic stages of Plasmodium falciparum and is not recognized by antibodies recognizing the blood stage of Plasmodium falciparum.

21. The nucleic acid sequence according to claim 18, wherein said nucleic acid sequence encodes an amino acid sequence of between 10 and 87 consecutive amino acids of sequence:

Glu-Phe-Arg-Val-Ser-Thr-Ser-Asp-Thr-Pro-Gly-Gly-Asn-Glu-
Ser-Ser-Ser-Ala-Ser-Pro-Asn-Leu-Ser-Gly-Ala-Arg-Glu-Lys-Lys-Asp-Glu-Lys-Gl
u-Ala-Ser-Glu-Gln-Gly-Glu-Glu-Ser-His-Lys-Lys-Glu-Asn-Ser-Gln-Glu-Ser-Ala-A
sn-Gly-Lys-Asp-Asp-Val-Lys-Glu-Glu-Lys-Lys-Thr-Asn-Glu-Lys-Lys-Asp-Asp-Gly-
Lys-Thr-Asp-Lys-Val-Gln-Glu-Lys-Val-Leu-Glu-Lys-Ser-Pro-Lys-Glu-Phe,

wherein said amino acid sequence is recognized by antibodies recognizing the sporozoite and hepatic stages of Plasmodium falciparum and is not recognized by antibodies recognizing the blood stage of Plasmodium falciparum.

22. The nucleic acid sequence according to claim 18, wherein said nucleic acid sequence encodes amino acid sequence

Glu-Phe-Arg-Val-Ser-Thr-Ser-Asp-Thr-Pro-Gly-Gly-Asn-Glu-Ser-Ser-Ser-Ala-Se r
-Pro-Asn-Leu-Ser-Gly-Ala-Arg-Glu-Lys-Lys-Asp-Glu-Lys-Glu-Ala-Ser-Glu-Gln-Gl
y-Glu-Glu-Ser-His-Lys-Lys-Glu-Asn-Ser-Gln-Glu-Ser-Ala-Asn-Gly-Lys-Asp-Asp-V
al-Lys-Glu-Glu-Lys-Lys-Thr-Asn-Glu-Lys-Lys-Asp-Asp-Gly-Lys-Thr-Asp-Lys-Val-
Gln-Glu-Lys-Val-Leu-Glu-Lys-Ser-Pro-Lys-Glu-Phe,

wherein said amino acid sequence is recognized by antibodies recognizing the sporozoite and hepatic stages of Plasmodium falciparum and is not recognized by antibodies recognizing the blood stage of Plasmodium falciparum.

23. The nucleic acid sequence according to claim 18, wherein said nucleic acid sequence encodes amino acid sequence

Ala-Arg-Glu-Lys-Lys-Asp-Glu-Lys-Glu-Ala-Ser-Glu-Gln-Gly-Glu-Glu-Ser-His-Ly s

-Lys-Glu-Asn-Ser-Gln-Glu-Ser-Ala,

wherein said amino acid sequence is recognized by antibodies recognizing the sporozoite and hepatic stages of Plasmodium falciparum and is not recognized by antibodies recognizing the blood stage of Plasmodium falciparum.

24. The nucleic acid sequence according to claim 18, wherein said nucleic acid sequence encodes amino acid sequence

Asn-Gly-Lys-Asp-Asp-Val-Lys-Glu-Glu-Lys-Lys-Thr-Asn-Glu-Lys-Lys-Asp-Asp-Gl y
-Lys-Thr-Asp-Lys-Val-Gln-Glu-Lys-Val-Leu-Glu-Lys-Ser-Pro-Lys-Glu-Phe,

wherein said amino acid sequence is recognized by antibodies recognizing the sporozoite and hepatic stages of Plasmodium falciparum and is not recognized by antibodies recognizing the blood stage of Plasmodium falciparum.

25. Recombinant DNA comprising a nucleic acid sequence according to claim 18, wherein said sequence is inserted into a heterologous host vector.

26. Recombinant DNA according to claim 25, wherein said nucleotide sequence is preceded by a promoter and followed by a signal sequence coding for the termination of transcription.

27. A DNA or RNA primer comprising 30 to 261 nucleotides of the nucleic acid sequence according to claim 20 or a sequence that hybridizes to the sequence according to claim 20 under conditions suitable for polymerase chain reaction (PCR).

28. A DNA or RNA primer comprising 30 to 261 nucleotides complementary to the nucleic acid sequence according to claim 20 or a sequence that hybridizes to the sequence according to claim 20 under conditions suitable for polymerase chain reaction (PCR).

29. A recombinant vector comprising

a recombinant DNA sequence, wherein said sequence comprises a nucleic acid according to claim 18; and

elements for expression of the peptide encoded by said nucleic acid sequence;

wherein said nucleic acid sequence and expression elements are inserted into a heterologous host vector at a site, which is not essential for replication of the host vector.

30. The recombinant vector according to claim 29, wherein said recombinant DNA further comprises a promoter, which precedes said nucleic acid sequence, and a signal sequence coding for the termination of transcription, which follows said nucleic acid sequence.

33. A method for the in vitro diagnosis of malaria comprising amplification of a nucleic acid according to claim 20 or a sequence that hybridizes to the sequence according to claim 20 under conditions suitable for polymerase chain reaction (PCR), employing

a DNA or RNA primer comprising 30 to 261 nucleotides of the nucleotide sequence according to claim 20 or a complement thereof or a sequence that hybridizes to the nucleotide sequence according to claim 20 under conditions suitable for polymerase chain reaction (PCR).

34. The method according to claim 33, comprising

(1) contacting a biological sample with said nucleic acid sequence under conditions permitting the formation of a hybridization complex between said nucleic acid sequence and nucleic acid sequences present in the biological sample; and

(2) detecting the formation of hybrid.

35. A diagnostic kit for the detection of a nucleic acid sequence according to claim 20 present in a biological sample, which nucleic acid sequence hybridizes with a DNA sequence to form a hybrid, wherein said kit comprises

a probe comprising a nucleic acid sequence according to claim 20;

a medium for carrying out a hybridization reaction between the nucleic acid sequence to be detected and said probe; and

a reagent to detect the hybrid formed between a nucleic acid sequence present in said biological sample and said nucleic acid sequence of claim 20;

wherein said DNA sequence, medium, and reagent are present in an amount sufficient to perform said detection.

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L10: Entry 49 of 78 File: USPT Aug 11, 1998

DOCUMENT-IDENTIFIER: US 5792609 A
TITLE: Detection of malaria

CLAIMS:

1. A nucleotide fragment consisting of a base sequence represented by the following sequence:

5'GAAAATTCCTTTCGGGGA3' (SEQ ID NO:1)

or the base sequence complementary thereto.

2. A nucleotide fragment consisting of a base sequence represented by the following sequence: ##STR4## or the base sequence complementary thereto.

3. A nucleotide fragment consisting of a base sequence represented by the following sequence:

5'GAGACATTCTTATATATG3' (SEQ ID NO:3)

or the base sequence complementary thereto.

4. A method of distinguishing falciparum malaria, tertian malaria, quartan malaria and ovale malaria from one another comprising hybridizing a nucleotide fragment consisting of a base sequence represented by one of the following sequences:

5'AAGTCATCTTTCGAGGTGAC3' (SEQ ID NO:4)

5'GAATTTTCTCTTCGGAGTTTA3' (SEQ ID NO:5)

5'GAGACATTCTTATATATG3' (SEQ ID NO:3)

5'GAAAATTCCTTTCGGGGA3' (SEQ ID NO:1)

5'CGACTAGGTGTTGGATGA3' (SEQ ID NO:6)

or the base sequences complementary thereto to a sample DNA in which falciparum malaria, tertian malaria, quartan malaria or ovale malaria is to be detected, detecting binding of said nuclecide fragment to said sample DNA, and correlating binding of said nucleotide fragment to said sample DNA to the presence of one of

falciparum malaria, tertian malaria, quartan malaria or ovale malaria.

5. Primers for detecting plasmodia each consisting of a nucleotide fragment consisting of the one of the base sequences represented by the following sequences:

5'GAACGAAAGTTAAGGGAGT3' (SEQ ID NO:7)

5'ACTGAAGGAAGCAATCTAA3' (SEQ ID NO:8)

5'TCAGTTACCGTCGTAATCTT3' (SEQ ID NO:9)

and

5'CCAAAGACTTTGATTTCTCAT (SEQ ID NO:10).

6. A primer or probe for detecting an ovale malaria parasite consisting of a nucleotide fragment comprising a base sequence represented by the following sequence:

5'GAAAATTCCTTTCGGGGA3' (SEQ ID NO:1),

the base sequence complementary thereto or a mutation sequence thereof, wherein said primer or probe specifically detects an ovale malaria parasite under the following conditions:

(A) amplification in 100 mM Tris-HCl (pH 8.9), 1.5 mM MgCl.sub.2, 80 mM KCl, 500 .mu.g/ml BSA, 0.1% (w/v) sodium cholate, 0.1% Triton X-100, 200 .mu.g/ml of proteinase K, 0.45% Tween 20 and 0.45% NP40 under conditions of 94.degree. C. for 30 seconds, 50.degree. C. for 60 seconds, and 72.degree. C. for 60 seconds; or

(B) hybridization in 5.times.SSC at 60.degree. C. for 1 hour.

7. A primer or probe for detecting an ovale malaria parasite consisting of a nucleotide fragment comprising a base sequence represented by the following sequence: ##STR5## or the base sequence complementary thereto or a mutation sequence thereof, wherein said primer or probe specifically detects an ovale malaria parasite under the following conditions:

(A) amplification in 100 mM Tris-HCl (pH 8.9), 1.5 mM MgCl.sub.2, 80 mM KCl, 500 .mu.g/ml BSA, 0.1% (w/v) sodium cholate, 0.1% Triton X-100, 200 .mu.g/ml of proteinase K, 0.45% Tween 20 and 0.45% NP40 under conditions of 94.degree. C. for 30 seconds, 50.degree. C. for 60 seconds, and 72.degree. C. for 60 seconds; or

(B) hybridization in 5.times.SSC at 60.degree. C. for 1 hour.

L12: Entry 19 of 33 File: USPT Oct 15, 1996

US-PAT-NO: 5565327
DOCUMENT-IDENTIFIER: US 5565327 A

TITLE: Methods of diagnosing parasitic infections and of testing drug susceptibility of parasites

DATE-ISSUED: October 15, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Greenleaf; Arno L.	Durham	NC		
Lee; Jae M.	Durham	NC		
Hardin; Steven E.	Louisville	KY		

US-CL-CURRENT: 435/21; 435/24, 435/34, 436/811

CLAIMS:

That which is claimed is:

1. A method of detecting blood-borne Plasmodium in a mammalian subject, comprising the steps of:

(1) collecting a sample containing red blood cells from a mammalian subject;

(2) treating said sample to lyse red blood cells and any Plasmodium present in said sample;

(3) contacting said sample with (a) a Plasmodium C-terminal domain (CTD) kinase substrate and (b) a phosphorus donor;

(4) detecting hyperphosphorylation of said substrate; and

(5) correlating the presence of hyperphosphorylation with the presence of blood-borne Plasmodium in said mammalian subject.

2. The method of claim 1, wherein said phosphorus donor is selected from the group consisting of adenosine triphosphate (ATP), uridine triphosphate (UTP), guanosine triphosphate (GTP), cytidine triphosphate (CTP), adenosine diphosphate (ADP), uridine diphosphate (UDP), guanosine diphosphate (GDP), and cytidine diphosphate (CDP).

3. The method of claim 1, wherein said phosphorus donor comprises radiolabelled phosphorus.

4. The method of claim 1 wherein said blood-borne Plasmodium is selected from the group consisting of Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale, and Plasmodium berghei.

5. The method of claim 1 wherein said Plasmodium C-terminal domain (CTE) kinase substrate is selected from the group consisting of:

a peptide of amino acid sequence (Tyr-Ser-Pro-Thr-Ser-Pro-Lys).sub.n (SEQ ID NO:2);

a peptide of amino acid sequence (Tyr-Ala-Pro-Thr-Ala-Pro-Lys).sub.n (SEQ ID NO:5); and

a peptide of amino acid sequence (Tyr-Ser-Pro-Thr-Ser-Pro-Arg).sub.n (SEQ ID NO:6);

where n is from one to one hundred.

L12: Entry 20 of 33 File: USPT Oct 8, 1996

DOCUMENT-IDENTIFIER: US 5563256 A
TITLE: Eimeria tenella 16S rDNA probes

Detailed Description Text (4):
An assay of this type must be sufficiently sensitive so as to be able to detect the expansion or reproduction of a very small oocyst inoculum. In other models DNA hybridization probes have been successfully used to quantitate parasite load in infected hosts. For example, exoerythrocytic forms (EEF) of Plasmodium berghei have been assayed in genomic DNA prepared from rat liver extracts using a repetitive plasmodial DNA probe (Ferreira et al., Mol. Biochem. Parasitol. 19:103-109 [1986]). More recently, oligonucleotide probes derived from rRNA sequences have been employed to quantitate EEF of Plasmodium yoelii in RNA prepared from the livers of infected mice (Arreaza et al., Exp. Parasitol. 72:103-105 [1991]). Similarly, any assay for a live coccidiosis vaccine must be capable of detecting Eimeria sequences contained within a total nucleic acid preparation (whether it be RNA or DNA) from chicken intestinal epithelia and mucosa. Because the Eimeria sequences represent such a small percentage of the genetic information in the extract, direct hybridization to DNA is not sufficiently sensitive to detect the vaccine oocyst dose for each species. Because of the biological amplification of rRNA sequences within cellular RNA pools, hybridization of the Eimeria species-specific oligonucleotide probes to RNA preparations from intestinal epithelia and mucosa is one way in which this assay and oligonucleotide probes of this invention can be used. Genomic DNA prepared from oocysts shed in the feces of vaccinated birds can also be characterized as a hybridization target for the oligonucleotide probes. This source of parasite nucleic add is relatively free of host genetic material and so in effect represents an enriched hybridization target.

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L12: Entry 30 of 33 File: USPT Apr 10, 1990

DOCUMENT-IDENTIFIER: US 4915941 A
TITLE: Method for preventing the development or decreasing the extent of malarial parasitemia

Detailed Description Text (11):
The DNA content of each blood sample was immobilized in 1.5 cm diameter, 0.45 millimicron pore-size nitrocellulose filters (Millipore Corp., Bedford, MA) and hybridized with a genomic 2.2 kb repetitive P. berghei DNA probe (obtained as described under "Preparation of DNA Probe", below), labelled with .sup.32 P by nick translation. To calculate background levels, filters containing (a) DNA from normal mouse blood and (b) carrier rat DNA were included in the hybridization assays. A standard hybridization curve was constructed by probing 10-fold dilutions of blood-stage P. berghei DNA. A linear relationship between the radioactivity associated with the filters and the amount of parasite DNA immobilized was always obtained in the range of 100 picograms to one microgram P. berghei DNA (Preparation of DNA Probe and FIG. 4). The amounts of the parasite DNA associated with the filters were calculated by reference to the standard curve and expressed as the means and standard deviations of the nanograms of P. berghei DNA detected in five animals. These values are represented in FIG. 1 in a logarithmic scale. Blood smears were prepared to measure parasitemia (a total of 10.sup.4 per slide were counted). The percent decrease in parasitemia was calculated from the hybridization experiment, by comparing the values obtained for the mammals pretreated with recombinant murine gamma-interferon with those obtained in placebo-treated mammals. The results are shown in FIG. 1.

Detailed Description Text (20):
Forty-four hours after sporozoite infection, the rats were bled from the axillary vein and artery and their livers were removed. DNA from these organs was purified and probed for the presence of P. berghei DNA. Values, expressed as means and standard deviations, represent the percent inhibition of EEF development and were obtained by comparing the amount of hepatic parasite DNA present in interferon- and placebo-treated groups.

Detailed Description Text (42):
Forty- to fifty-day old female Norway Brown rats (Harlan Sprague Dawley, Indianapolis, IN) were bled from the axillary vein and artery. Livers were perfused with cold PBS, removed and frozen in liquid N.sub.2. To purify the DNA, livers were homogenized in the presence of 150 mM NaCl, 10 mM EDTA, 1% w/v SDS, followed by the addition of sodium perchlorate to 0.5M. Two organic solvent extractions were performed, the first with 1 volume of a chloroform-isoamyl alcohol mixture, and the second with 1 volume of a chloroform-isoamyl alcohol-phenol mixture. The DNA in the aqueous phase was ethanol-precipitated, spooled onto a glass rod, dissolved in water, treated with RNAase (Boehringer Mannheim, Indianapolis, IN), alpha-amylase (type II-A, Sigma Chemical Company, St. Louis, MO) and proteinase K (BRL, Gaithersberg, MD), reprecipitated with ethanol, redissolved in water, and the concentration was measured at 260 mM. In order to obtain blood stages of Plasmodium berghei DNA, female A/J mice (Jackson Laboratories, Bar Harbor, ME) were injected with

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Search Results - Record(s) 1 through 1 of 1 returned.

L13: Entry 1 of 1 File: USPT Nov 17, 1987

US-PAT-NO: 4707445
DOCUMENT-IDENTIFIER: US 4707445 A

TITLE: Intact gene and method of excising and cloning same

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Detection of DNA sequences in *Plasmodium berghei* by means of in situ hybridization

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Summary. A non-radioactive in situ hybridization technique, used to map unique DNA sequences to plant chromosomes, has been adapted for the localization of specific DNA sequences in nuclei of *Plasmodium berghei*. After hybridization using probes labeled with biotin-11-dUTP, the formed DNA/DNA hybrids were detected by fluorescence microscopy using a specific double-layer antibody technique. Besides its high resolution, this procedure is characterized by a high sensitivity, allowing the detection of a unique sequence as small as 2.5 kb.

Introduction

A central point within the biology of malaria parasites is the development of sexual stages, the gametocytes (Baker 1989). Recently, the interest in the molecular biological analysis of this process has strongly increased. When suitable nucleic acid sequences are isolated, a sensitive in situ hybridization procedure will allow the detection of specific DNA sequences and/or messenger RNA in individual cells. If sensitive enough, such a technique would allow identification of the actual induction of the sexual process within the life cycle of malaria parasites. Various procedures are available today to obtain specific DNA/DNA or DNA/RNA hybrids with labels that can be detected by immunological marking techniques (Wilchek and Bayer 1988).

We have adapted a sensitive in situ hybridization technique for *Plasmodium berghei*. This technique is based on the incorporation of a biotinylated analogue of dTTP (bio-11-dUTP), into the probe DNA by nick-translation. This procedure was previously used to map a unique, 17 kilobases (kb), DNA sequence to plant chromosomes (Ambros et al. 1986a, b). In our experiments, the formed hybrids were detected by a specific double-layer fluorescent antibody technique described by Albertson (1984). The combination of these two techniques could be successfully used for the immunocytochemical detection of in situ hybridized biotinylated probes in *P. berghei*.

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Materials and methods

Parasites. Cloned *P. berghei* lines 227L and 213L derived from the ANKA isolate, described by (Cornelissen et al. 1985), were used. *Trypanosoma brucei* 427, the trypanosomal stock used for these experiments has been described by Cross (1975).

Preparation of microscopic slides. Slides were precleaned as described by van Prooijen-Knegt et al. (Van Prooijen-Knegt et al. 1982) and stored in 0.02% NaN_3 prior to use. Bloodsmears were obtained from the tail of *P. berghei*-infected mice according to standard procedures. Smears were air-dried and fixed in methanol/acetone (1:2 v/v) for 4 min at −20° C followed by air drying (Cornelissen et al. 1983).

Hybridocytochemistry. Hybridization was performed essentially as described by Ambros et al. (1986a, b), and Ambros and Schweizer (1987). Our initial experiments were performed with the aim to determine the influence of the following parameters on the signal strength and nuclear morphology in *P. berghei*: (1) fixation time, (2) denaturing conditions (temperature, time), (3) hybridization mixture (SSC concentration, formamide percentage, amount of carrier DNA), (4) hybridization (temperature, time), and (5) post-hybridization washes. This resulted in modifications of the procedures mentioned above, to obtain an optimisation of the signal-to-noise ratio (Obst 1989).

The optimized procedure consists of the following steps: microscopic preparations were incubated with RNase A (100 µg/ml) in 2 × SSC (1 × SSC = 0.15 *M* NaCl, 15 m*M* sodium citrate, pH 7.0) for 1 h at 37° C to remove endogenous RNA. After two rinses in 2 × SSC for 20 min at room temperature to remove the RNase A, slides were dehydrated through a graded series of ethanol and air-dried. Slides were covered with 25 µl hybridization mixture under a precleaned coverslip and sealed with rubber cement. Chromosomal DNA and probe were denatured simultaneously in the hybridization mixture for 5 min at 71° C and hybridization was performed at 39° C overnight in a humid chamber. The hybridization mix contained 50% deionized formamide, 2 × SSC, 5% dextran sulphate, 200 ng/µl sonicated salmon sperm DNA and 2.5 ng/µl probe. The rubber cement was removed with an forceps and the coverslips were removed by immersing the slides in 2 × SSC

at room temperature for 10 min, followed by washes in 2 × SSC (2 × 10 min each) at 39° C, 2 × SSC (5 min), PBS (0.13 *M* NaCl, 3 m*M* NaH_2PO_4, 7 m*M* Na_2HPO_4)/0.1% Triton X-100 (3 min) and PBS (3 × 5 min each) all at room temperature.

Synthesis of biotinylated probes. Three different probes were used. The probe pPb2.3 encodes one telomeric repeat unit on a 2.3 kb HindIII fragment cloned into pUC19 (J. Haller, unpublished results) and is similar to pPbT2.3 first described by Ponzi et al. (1985). The telomeric unit is present in 300 copies per genome (Pace et al. 1987). The probe pPbSL7.8 contains a 7.8 kb EcoRI fragment (Dame and McCutchan 1983), encoding the small and part of the large rRNA genes of *P. berghei* and is present at 4 copies per haploid genome (Dame and McCutchan 1983). The recombinant clone pPb2.5 contains a 2.5 kb HindIII fragment cloned into pUC19 (J. Haller, unpublished results). This is a unique, random cloned sequence with unknown function (Obst 1989). Either the entire plasmid or their respective inserts were used as DNA probes. Plasmid and insert DNA were isolated according to standard procedures ((Evers et al. 1989) and cited references). DNA probes were labeled by nick translation (Rigby et al. 1977) with Bio-11-dUTP (Gibco/BRL).

Signal detection. For the detection of biotin-labeled probes, all procedures following the wash step were carried out essentially as described by Albertson (1984). The method was, however, slightly modified to optimise the signal detection in *P. berghei* (Obst 1989). Briefly, slides were washed in PBS/0.5% Tween 20 for 10 min to prevent unspecific binding of antibodies. Immediately thereafter, slides were incubated with rabbit anti-biotin (IgG fraction, ENZO) at a final dilution of 1:100 in PBS/2% BSA (bovine serum albumin) for 1 h at 37° C in a humid chamber, then rinsed in three changes of PBS (5 min each); the final wash contained also 0.5% Tween 20. Subsequently, TRITC (tetramethyl rhodamine)-labeled goat anti-rabbit antibodies (Dunn) were added using the same dilution, incubation time and wash procedure as described for the anti-biotin antibody. The slides were incubated in PBS containing 1 μg/ml DAPI (4′-6′ diamidino-2 phenylindole) to stain the nuclear DNA (Cornelissen et al. 1983). The slides were rinsed briefly in PBS (3 × 30 s each) and sealed with 300 μl mounting solution under a coverslip. The mounting solution consisted of 9 parts glycerol and 1 part 0.2 *M Tris*/HCl pH 8, containing 2% DABCO (1,4 Diazabicyclo(2.2.2)octane (Sigma)) and 0.02% NaN_3 (Johnson et al. 1982); DABCO was added to prevent fading of fluorescence.

Microscopy. The slides were examined with a Nikon Microphot-FX microscope equipped with an epi-illuminator, an oil immersion objective (Plan Apo 100/1.40), and a HBO 100 W mercury arc. To visualize the fluorescence, the following filters were used: UV-2A for UV excitation (DAPI counterstain) and G-2A for green excitation (TRITC). Photographs of fluorescing objects were taken with a FX-35A camera body (Nikon) on Ilford HP5 film.

Results

General considerations

The biotin-labeled probes were hybridized to microscopic preparations of *P. berghei* using conditions determined to be optimal for in situ hybridization with malarial parasites as described under 'Materials and methods'. The formed hybrids were detected with a double-layer fluorescent antibody technique, based on rhodamine fluorescence. After the hybridization procedure the DNA of the *P. berghei* cells was counterstained with DAPI. Representative photographs of the same field were made of the blue DAPI and of the red rhodamine fluorescence. The results of the in situ hybridization experiments are shown in Fig. 1.

A series of control experiments proved conclusively that the in situ hybridization reaction was detecting malarial sequences specifically. No hybridization signal was obtained when *P. berghei* cells were incubated with the hybridization solution alone, with unlabeled probe, or with an biotinylated plasmid followed by the detection assay (data not shown and Fig. 1 A). Similarly, no signal was obtained when *P. berghei* biotinylated probes were hybridized to nuclei and kinetoplasts of the protozoan parasite *T. brucei* (Fig. 1 B). Moreover, the malarial probes did not cross-hybridize to the nuclei of mouse white blood cells, which were present on the same slide (Fig. 1 D). However, nuclei of *P. berghei* cells stained with biotinylated malarial telomeric and rDNA probes clearly stood out over the background (Fig. 1 C–F). These data clearly show that only the biotinylated malarial probes resulted in a specific signal with high resolution over *P. berghei* nuclei (Fig. 1).

Hybridization signal as a function of the probe

The probes used in the experiments described below differ both in fragment size and copy number. The availability of these probes, ranging from 2.5–690 kb per *P. berghei* nucleus, allowed us to determine the sensitivity of the developed methods described above. The signals obtained with the biotin-labeled inserts of the telomeric (pPb2.3) and rDNA (pPSL7.8) clones were visible as a number of bright dots in all nuclei examined (data not shown).

In an attempt to enhance the signal further, we labeled not only the insert, but the whole plasmid. The advantage of this approach is an increase of the DNA network formed during hybridization. The insert will specifically hybridize to the nuclear DNA of *P. berghei* and, since the plasmid is randomly cleaved during the nick-translation reaction, also form hybrids with the vector sequences. Vector/vector hybrids will subsequently form an extensive DNA network, thereby increasing the number of biotin-molecules significantly at the specific site (cf. Harper and Saunders 1981). The data shown in Fig. 1 are all obtained with *P. berghei* probes labeled in this way.

We were unable to correlate the number of the fluorescent dots generated by the telomeric probe (Fig. 1 C, D) to the chromosome number of *P. berghei* ($n = 14$; Janse et al. 1989). The number of fluorescing dots per nucleus was in all preparations significantly less than the actual chromosome number of *P. berghei*. The distribution of the rDNA probe was confined to a limited number of fluorescing dots, approximately 3–6 per nucleus (Fig. 1 E). This observation is in agreement with the copy number of the rDNA unit, which is present as four copies per nucleus (Dame and McCutchan 1983 a, b). Finally, the rDNA signal was not confined to a single site within the nucleus, which indicates the absence of a nucleolus. Our observations are in agreement with a previous analysis of malaria nuclei after

Feulgen staining, in which a small nucleolus was occasionally observed in gametocytes (Cornelissen et al. 1984a).

We noticed that the signal obtained with the rDNA probe was much stronger than that obtained with the telomeric probe (Fig. 1C, D vs. 1E), not withstanding the fact that the other sequence represents approximately 20-fold more DNA sequence within the *P. berghei* genome. The difference in fluorescence intensity could be merely due to a marked difference in the biotin labeling of either the rDNA or telomeric probe, or a difference in the AT-content of both probes. We can, however, exclude both possibilities. Firstly, we verified the biotin labeling of both probes used in the experiments by spot blots (Obst 1989). After independent nick-translation reactions, using both probes, 0.3–1.0 pg of biotin-labeled DNA could be detected in spotblots indicating that both probes are labeled to the same extent. Secondly, the telomeric probe has a high AT content, namely 78% (Pace et al. 1987), which might influence the hybrid stability. To verify whether the temperature (39° C) in the standard hybridization protocol (see 'Materials and methods') could have a major effect, we evaluated the influence of the hybridization temperature. An increase to 42° C resulted in a decrease of the fluorescence signal whereas a decrease to 36.5° C resulted in a comparative fluorescence signal as obtained at 39° C. However, a lowering of the hybridization temperature to 36.5° C resulted in an increase of the non-specific background. Finally, since rDNA (Dame and McCutchan 1983a, b) is transcribed at a much higher level than the telomeric sequences (Rudenko and Van der Ploeg 1989), the difference could be due to the formation of RNA/DNA hybrids. This is, however, rather unlikely because of the excessive RNase treatment of the preparations prior to the actual DNA hybridization in the standard protocol.

The insert of the recombinant clone pPb2.5, which is a single copy fragment, did not result in a reproducible positive signal. However, when insert and vector were biotinylated and used as a probe a weakly positive, but reproducible, signal over 15–40% of the *P. berghei* nuclei could be detected. The signal was always confined to a single spot within the malarial nucleus (Fig. 1F).

Discussion

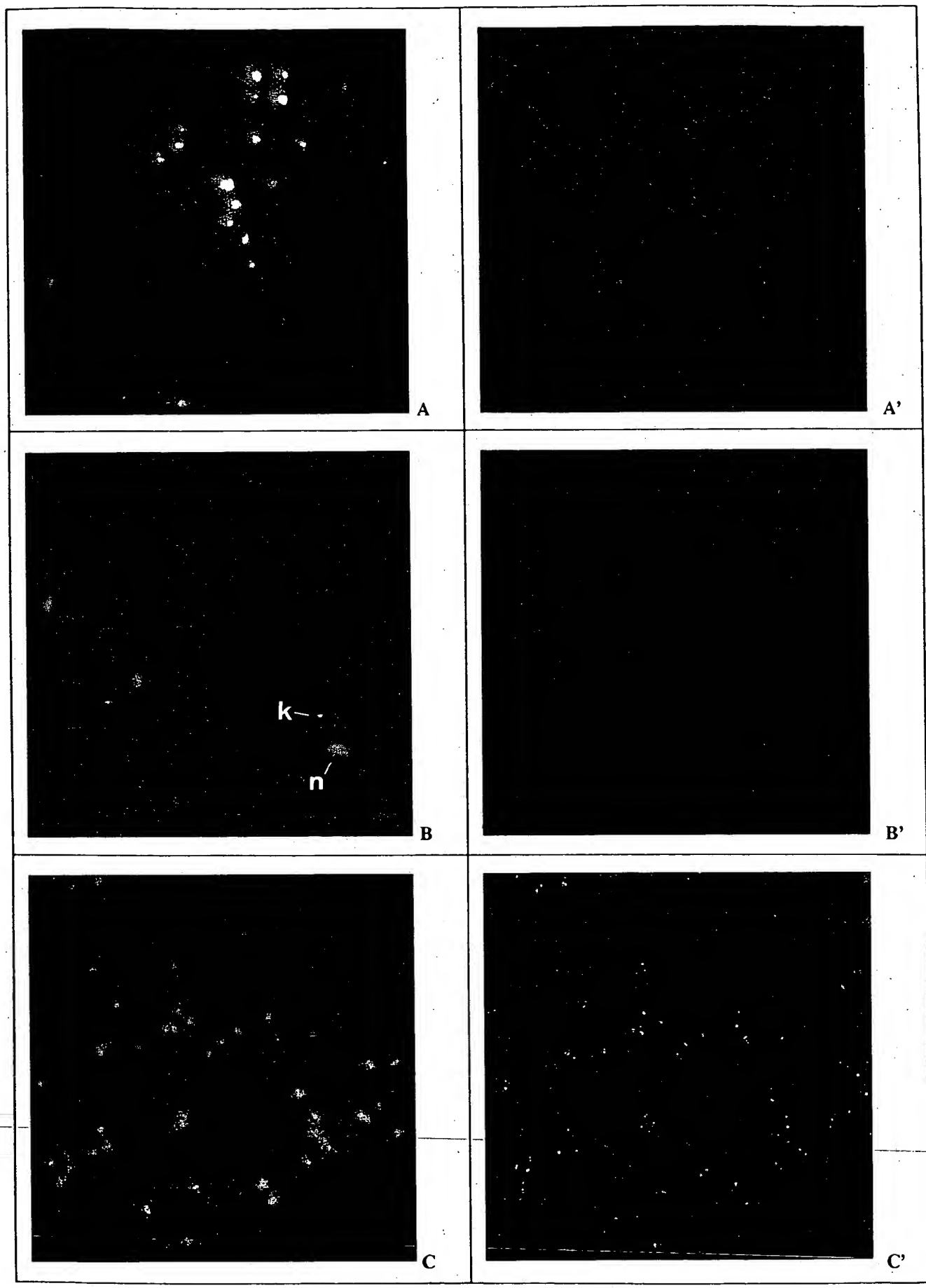
We performed in situ hybridization of biotin-labeled DNA probes to nuclear DNA on microscopic preparations of *P. berghei*. The hybrids were detected by an antibody double-layer assay using rhodamine-labeled second antibodies and fluorescence microscopy. The conditions employed resulted in the detection of telomeric and rDNA sequences in *P. berghei* nuclei with low backgrounds. The signal could, however, be enhanced by using the insert as well as vector sequences (cf. Ambros et al. 1986b). The telomeric and rDNA probes represent approximately 690 kb and 31.2 kb of DNA sequence which is, respectively, 3% and 0.14% of the *P. berghei* genome (Cornelissen et al. 1984b). Therefore, these sequences do not allow a qualitative evaluation of the sensitivity of the technique employed.

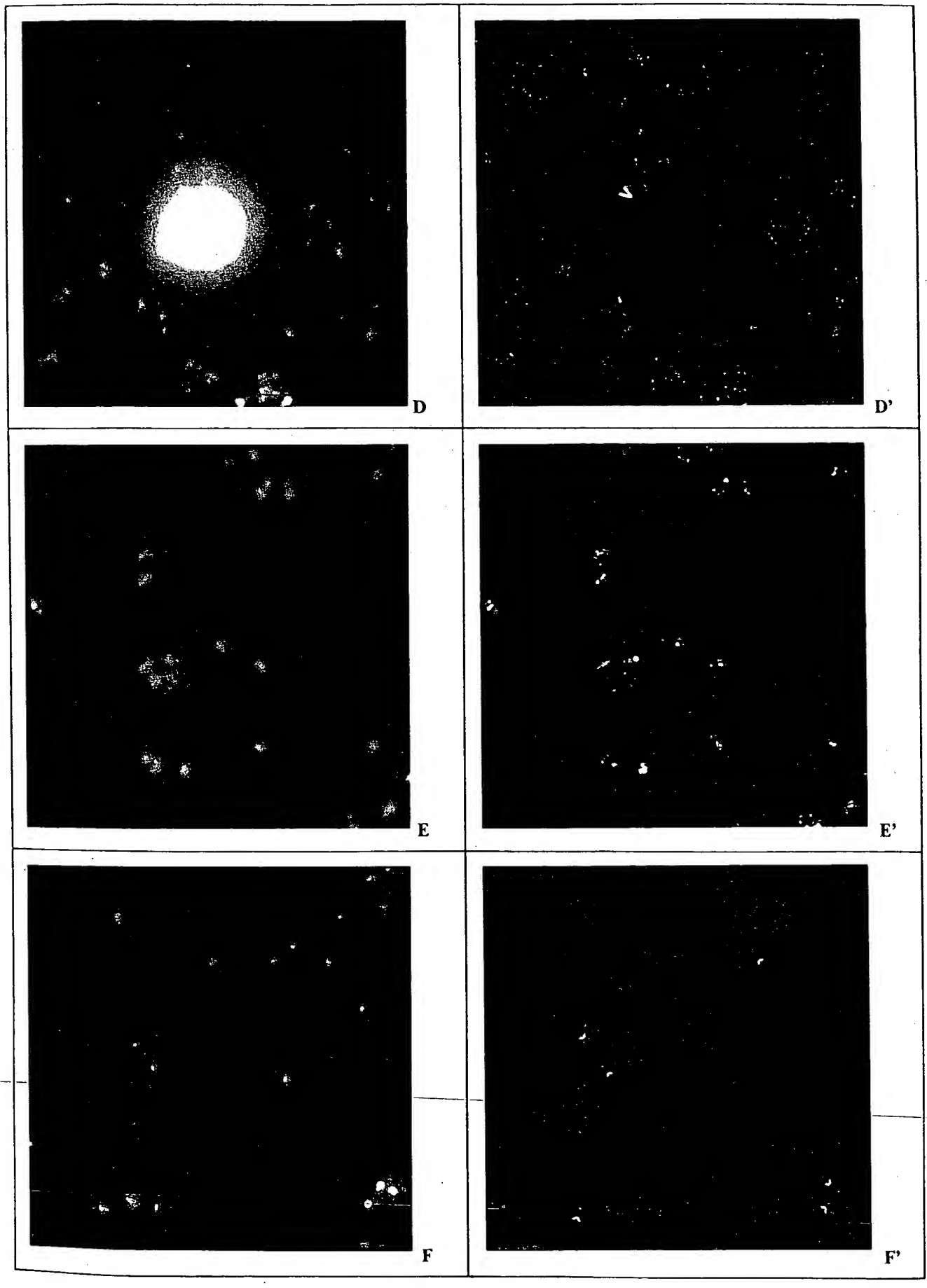
Since we have optimized the in situ hybridization procedure and sequences of 2–3 kb can be detected with the original technique (Ambros and Schweizer 1987), we have evaluated whether it was possible to detect a single copy sequence of 2.5 kb in *P. berghei*. When the whole plasmid pPb2.5 was used, a positive signal in 15–40% of the nuclei was obtained. The qualitative sensitivity of our procedure is in agreement with other published procedures using biotinylated DNA probes in which 20–70% of the cells or chromosomes showed a positive signal using probes of 1 kb (Garson et al. 1987), 5 kb (Lawrence et al. 1988) and 17 kb (Ambros et al. 1986b). The most important factors determining the fact that only a percentage of the cells shows a positive signal when small probes are used, are the accesibility of the DNA for the probe (Pinkel et al. 1986) and the retention of the target sequences during the in situ hybridization procedure. DNA loss during in situ hybridization procedures can reach up to 40% of the total DNA present on microscopic slides (Raap et al. 1986). It is interesting to note here, that the sensitivity reached with biotin-labeled probes is similar to the sensitivity reached by other non-isotopic detection methods (Hopman et al. 1986; Landegent et al. 1985).

Preliminary data, based on direct measurements of nuclear DNA, suggest that some form of DNA amplification might be involved during the differentiation process of macrogametocytes (reviewed in (Cornelissen 1988; Frontali 1985; Janse and Mons 1987)). Since the hybridization technique described in this paper allows the detection of DNA probes of 2.5 kb in size, the method can actual be applied to analyse this process when suitable DNA probes have been identified.

We observed that the fluorescence intensity of the telomeric probe was relatively weak in relation to the total size of the DNA segments encoding this sequence and in comparison to the signal obtained with the rDNA probe. We could exclude that this observation was due to simple artefacts such as: (1) a difference in biotin labeling of the probes used, (2) an effect of hybrid stabili-

Fig. 1. Photographs of cells in which the biotin-labeled DNA hybrid is visualized with TRITC immunofluorescence, mounted and counterstained with DAPI. Parallel photographs of the same field, showing first the DAPI fluorescence and then the TRITC fluorescence, are indicated with the same letter, without and with an accent, respectively. (**A**) Control experiments in which *P. berghei* cells were hybridized with a biotinylated plasmid without insert. (**B**) *T. brucei* cells hybridized with the biotinylated telomeric sequence probe (pPb2.3) of *P. berghei*. The DAPI fluorescence is localized both over the nuclei (*n*) and kinetoplasts (*k*) of the trypanosomes. (**C**) *P. berghei* cells to which the biotinylated telomeric probe pPb2.3 is hybridized. (**D**) The same experiment as described in (**C**), except that here a mouse white blood cell is present (*arrowhead* in **D'**). (**E**) *P. berghei* cells to which the biotinylated recombinant rDNA clone pPbSL7.8 was hybridized. (**F**) *P. berghei* cells to which the biotinylated recombinant clone pPb2.5 was hybridized. Only part of the nuclei show a weak positive signal (*arrowheads* in **F'**)


A
A'
k
n
B
B'
C
C'


D
D'
E
E'
F
F'

ty due to a difference in AT-content of both probes, and (3) the formation of fortuitous RNA/DNA hybrids. Since it is likely that non-specific DNA loss during the in situ hybridization procedure will be sequence independent, the difference is most likely due to a reduced accessibility of the telomeric probe. Two arguments in line with this reasoning can be easily found. Firstly, the level of transcription of the rDNA- and telomeric sequence might influence the accessibility of their respective probes. The high transcription rate of the rRNA genes might result in a more open DNA configuraton and therefore be more optimal for DNA/DNA hybridization. Although the telomeric sequences are transcribed (Rudenko and van der Ploeg 1989), the transcription level is relatively low and the DNA sequence might therefore be less accessible to the telomeric probe. Secondly, although the telomeric sequences are also found in chromosome-internal positions (Pace et al. 1987), the telomeric sequence present in telomeres might have a reduced accessibility due to the attachment of telomeres to the nuclear matrix.

In conclusion, the method presented here allows a rapid and unambiguous localisation of specific DNA sequences in *P. berghei* nuclei. The method is characterized by a high resolution and high sensitivity and should, in principal, also be applicable to other representatives of the *Haemosporina*. A minor modification of the method described in this paper might allow the detection of specific transcripts in malaria parasites, since biotin-labeled probes have also been used to monitor gene expression by detecting specific mRNAs in higher eukaryotes (Lawrence and Singer 1985, 1986; Zabel and Schäfer 1988). Finally, the method can be easily combined with a sandwich assay, using FITC-labeled secondary antibodies, directed against stage-specific surface or cell-internal epitopes of *P. berghei* (Obst 1989), resulting in a positive discrimination of the stages within the life cycle of the malaria parasite. This identification is a prerequisite to allow an analysis of stage-specific gene expression in malaria parasites by in situ hybridization.

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TABLE 1

Sequences of the Adaptors Used in the Construction of the pGBT9 and pGAD424 Vectors in All Six Reading Frames

Adaptor	Sequence
+1	5′-AATTTGGAATTCCCGGG-3′ 3′-ACCTTAAGGGCCCCTAG-5′
+2	5′-AATTGAATTCCCGGG-3′ 3′-CTTAAGGGCCCCTAG-5′
GBT-rev	5′-AATTTGCGCTGCAGGTCGACGGATCCCCGGGAATTCTTGCA-3′ 3′-ACGCGACGTCCAGCTGCCTAGGGGCCCTTAAGA-5′
GAD-rev	5′-AATTTGCGAGATCTCTGCAGGTCGACGGATCCCCGGGAATTCT-3′ 3′-ACGCTCTAGACAGCTGGACCTGTCCAGGGGCTCCTAAGACTAG-5′

Note. The adaptors +1 and +2 were used to construct the vectors pGBT9(+1), pGBT(+2), pGAD424(+1), and pGAD424(+2). The adaptor GBT-rev was used to construct the plasmids pGBT9rev, pGBT9(+1)rev, and pGBT9(+2)rev, whereas the adaptor GAD-rev was used for the construction of pGAD424rev, pGAD424(+1)rev, and pGAD424(+2)rev.

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A One-Step Lysis Procedure for 18S Ribosomal RNA-Based Diagnosis of Infection by *Plasmodium* Species

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Comparative analysis of the 18S rRNA sequences of the malaria parasites (1–3) has revealed distinct regions which are species specific. The potential use of such sequences for the design of species-specific probes has been shown (4–6). The ultimate goal of any diagnostic system which focuses on parasite detection is to attain a level of simplicity which would enable it to be used under field conditions. A very important component of this process involves the development of simple single-step procedures which would enable the release of detectable target. In the case of complex biological fluids such as blood, there should be minimum retention of blood protein or color for subsequent nonradioactive adaptation. Finally, any such procedure should not involve the use of any corrosive or toxic chemicals. We report here the development of a simple, single-step lysis procedure for the detection of malaria parasites. The procedure has been evaluated using oligonucleotide probes targeted to the parasite 18S rRNA in both laboratory and limited field settings.

Materials and methods. Plasmodium berghei (NK strain) was maintained in an outbred ICR strain of mice. Parasitemia counts were based on tail tip smears. Blood reconstituted to varying degrees of parasitemia was mixed with 4 vol of lysis buffer containing Tris, varying amounts of EDTA with or without proteinase K, SDS, NP-40, or Sarkosyl. The lysed material was applied to membrane supports using conventional slot blot apparatus. *Plasmodium falciparum* strains were maintained in culture by the procedure described by Trager and Jensen (7). Culture material of known parasitemia was reconstituted in uninfected human blood and lysed.

Finger-prick blood was collected from reported fever cases from an endemic area in India. Samples were evaluated microscopically using Giemsa-stained thin blood smears. Required volumes of blood were processed according to the simplified lysis procedure adopted. For simplified lysis 10 μl of culture material reconstituted in uninfected blood was added to 10 μl of the lysis mixture. The lysate was hand dotted onto positively charged nylon membranes which were subsequently air-dried, gently agitated in 0.05% tetrasodium

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Primary sequences of two small subunit ribosomal RNA genes from Plasmodium falciparum.
McCutchan T F; de la Cruz V F; Lal A A; Gunderson J H; Elwood H J; Sogin M L
Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD.
Molecular and biochemical parasitology (NETHERLANDS) Feb 1988, 28 (1) p63-8, ISSN 0166-6851 Journal Code: 8006324
Contract/Grant No.: 6M32964; PHS
Document type: Journal Article
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tRNA genes transcribed from the plastid -like DNA of Plasmodium falciparum.
Preiser P; Williamson D H; Wilson R J
Division of Parasitology, National Institute for Medical Research, Mill Hill, London, UK.
Nucleic acids research (ENGLAND) Nov 11 1995, 23 (21) p4329-36, ISSN 0305-1048 Journal Code: 0411011
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Localization of ribosomal RNA and Pbs21-mRNA in the sexual stages of Plasmodium berghei using electron microscope in situ hybridization.
Shaw M K; Thompson J; Sinden R E
Molecular and Cellular Parasitology Group, Department of Biology, Imperial College of Science, Technology and Medicine, London/United Kingdom.
European journal of cell biology (GERMANY) Nov 1996, 71 (3) p270-6, ISSN 0171-9335 Journal Code: 7906240
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Localization of ribosomal RNA and Pbs21-mRNA in the sexual stages of Plasmodium berghei using electron microscope in situ hybridization.
Shaw M K; Thompson J; Sinden R E
Molecular and Cellular Parasitology Group, Department of Biology, Imperial College of Science, Technology and Medicine, London/United Kingdom.
European journal of cell biology (GERMANY) Nov 1996, 71 (3) p270-6, ISSN 0171-9335 Journal Code: 7906240
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Identification and quantification of rodent malaria strains and species using gene probes.
Snounou G; Bourne T; Jarra W; Viriyakosol S; Brown K N
Division of Parasitology, National Institute for Medical Research, Mill Hill, London.
Parasitology (ENGLAND) Aug 1992, 105 (Pt 1) p21-7, ISSN 0031-1820 Journal Code: 0401121
Document type: Journal Article
Languages: ENGLISH
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dna and cell biology, Weissig, V et al, 1997, Vol. 16(12), pages 1483-1492.

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Conserved location of genes on polymorphic chromosomes of four species of malaria parasites.
Janse C J; Carlton J M; Walliker D; Waters A P
Laboratory for Parasitology, University of Leiden, The Netherlands.
Molecular and biochemical parasitology (NETHERLANDS) Dec 1994, 68 (2) p285-96, ISSN 0166-6851 Journal Code: 8006324
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
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Infectivity of Plasmodium berghei sporozoites measured with a DNA probe.
Ferreira A; Enea V; Morimoto T; Nussenzweig V
Molecular and biochemical parasitology (NETHERLANDS) May 1986, 19 (2) p103-9, ISSN 0166-6851 Journal Code: 8006324
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Infectivity of *Plasmodium berghei* sporozoites measured with a DNA probe

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A 2.3 kb, ^{32}P-labeled repetitive DNA probe of *Plasmodium berghei* was used to measure the amount of parasite DNA in the liver of Norway Brown rats and mice infected with sporozoites. Standard hybridization curves were obtained by probing different amounts (100 pg to 1 μg) of *P. berghei* DNA immobilized on nitrocellulose filters. Host DNA did not interfere with hybridization specificity and sensitivity. A 100-fold increase in hepatic parasite DNA was detected between 25 h post-infection and the peak of parasite proliferation, detected at 44 h. The amount of parasite DNA increased with the number of injected sporozoites. At 5 h post-infection, a large proportion of parasite DNA was found in the spleen. However, this diminished with time and was negligible in amount at 25 h. A significant number of viable sporozoites were probably cleared in the spleen, since considerably more parasite DNA was found in the livers of splenectomized rats than in sham-operated counterparts. Although older rats develop much lower parasitemias upon inoculation of sporozoites, no significant differences were observed in the amount of parasite DNA in rats, 43 and 152 days old, injected with equal numbers of sporozoites. The higher resistance to malaria displayed by older rats is probably controlled by post-hepatic events. The infectivity of sporozoites for A/J mice was calculated to be about 1/20th that of Norway Brown rats.

Key words: *Plasmodium berghei*; Sporozoites; DNA probe; Exoerythrocytic forms

Introduction

The development of anti-sporozoite vaccines will require better methods to evaluate sporozoite infectivity, usually measured by estimating the 'prepatent period', that is, the time between sporozoite infection and the appearance of parasites in peripheral blood smears [1]. Although prepatent periods are inversely related to the size of the inoculum, the differences in the dose–response curves are very small, as illustrated in studies in which large numbers of rhesus monkeys were infected with varying doses of *Plasmodium cynomolgi* sporozoites [2]. For example, challenge with 10^4 or 10^7 sporozoites led to prepatent periods of 11.3 ±2.0 and 10.6 ±1.1 days, respectively.

Another method to measure the infectivity of sporozoites is to enumerate exoerythrocytic forms (EEF) by microscopic examination of liver sections. Although this approach has been useful for structural studies of exoerythrocytic development [3–5], quantitation is difficult.

Here we use a DNA probe, obtained by nick-translation of a cloned repetitive segment of DNA, to detect and measure liver-associated *P. berghei*. Using this probe we were able to study some of the factors which influence the number of developing EEF in rats such as, for example, the role of the spleen and the age of the animals.

Abbrevations: EDTA, ethylenediaminetetraacetic acid; EEF, exoerythrocytic forms; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SSC, sodium chloride, sodium citrate solution; SDS, sodium dodecyl sulfate.

Materials and Methods

Sporozoites. P. berghei sporozoites (NK65 strain),

maintained by cyclic passage of the parasite through *Anopheles stephensi* mosquitoes and hamsters, were collected 14–18 days after an infective blood meal by dissecting the salivary glands into Medium 199 containing 10% heat-inactivated normal rat serum [6] and injected into the dorsal tail vein or the portal vein of the animals. In the latter case, laparotomy was performed under anesthesia with 25 mg (kg body weight)$^{-1}$ Nembutal (Abbott Laboratories, North Chicago, IL).

The viability of different sporozoite batches was assessed by injecting 3 rats and 5 mice with 5000 sporozoites each. In all experiments the control groups became patent on the 4th or 5th day after infection. Viability was also tested by microscopic observation of circumsporozoite reactions following incubation with an anti-circumsporozoite protein monoclonal antibody [7,8].

Animals. 40–50 day old female Norway Brown rats were purchased from Harlan Sprague Dawley, Indianapolis, IN. 1–2 month-old A/J female mice were obtained from Jackson Laboratories, Bar Harbor, ME. Some animals were splenectomized by using standard surgical procedures and allowed to recover for at least a week before injection of sporozoites.

Preparation of the probe. A 2.3 kilobase pair (kb) probe (p263-1), isolated from a genomic library of *P. berghei* DNA prepared in the plasmid vector pBR322, represents a family of well-conserved repetitive sequences comprising about 3% of the parasite genome. Details on the isolation and characterization of the clone and the general method for the isolation and characterization of repetitive sequences will be reported elsewhere (V. Enea and D. Eichimper, in preparation; V. Enea, submitted).

Nick translation. All reagents were obtained from New England Nuclear (Boston, MA) and the assay was performed under standard conditions [9]. In a typical reaction mixture, 0.2 μg of pBR322 containing the repetitive *P. berghei* DNA segment inserted in the BamHI site, were nick-translated for 2 1/2 h at 13°C, in the presence of DNA polymerase, DNAase I, cold deoxynucleoside triphosphates and [α-^{32}P]dCTP. Free nucleotides were removed by filtering the reacti n mixture through coarse Sephadex G-50 (Pharmacia, Piscataway, NJ). Specific activities in excess of 4 × 10^8 cpm μg^{-1} of DNA template were routinely obtained.

Purification of rat liver and spleen DNA. Rats were bled from the axillary vein and artery. Livers were perfused with cold phosphate-buffered saline (PBS), removed and frozen in liquid N_2.

Purification of DNA followed standard procedures [10] with some modifications. Briefly, livers were homogenized in the presence of 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% w/v sodium dodecyl sulfate (SDS), followed by the addition of sodium perchlorate to 0.5 M. Two organic solvent extractions were performed, the first with a chloroform–isoamyl alcohol mixture and the second with a chloroform–isoamyl alcohol–phenol mixture. The DNA in the aqueous phase was ethanol-precipitated, spooled onto a glass rod, dissolved in water, treated with RNAase (Boehringer Mannheim, Indianapolis, IN), α-amylase (type II-A, phenylmethylsulfonyl fluoride (PMSF) treated, Sigma Chemical Co., St. Louis, MO) and Proteinase K (BRL, Gaithersburg, MD), reprecipitated with ethanol, redissolved in water, and the concentration measured at 260 nm. Purity of the preparations was assessed by determining the ratios of absorbance at 260 and 280 nm. Values equal or very close to 1.8 were routinely obtained. Purification of spleen DNA followed the same protocol, except that the α-amylase treatment was omitted.

Purification of P. berghei DNA of blood stages for the construction of standard curves. A/J female mice were injected with 5000 *P. berghei* sporozoites. 10 days after injection the animals were bled in the presence of heparin. The blood was spun down and the buffy coat removed. The pellet was washed, resuspended and passed through a glass wool column. By microscopic observation no contamination with white cells was detected in the effluent. The purified red cells were treated with 0.015% (w/v) Saponin (Sigma, St. Louis, MO) and incubated at 37°C for 15 min. The re-

leased parasites were washed with PBS, homogenized in the presence of 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 mM EDTA and 0.5% SDS. After treatment with Proteinase K, this material was twice extracted with a chloroform–isoamyl alcohol–phenol mixture and precipitated with ethanol. This was followed by a second cycle of Proteinase K–RNAase treatments, organic solvent extractions and ethanol precipitations. The pellet was dissolved in water and the DNA concentration measured. The usual recovery from 10^8 infected cells (mostly infected with rings and trophozoites) was approximately 10 μg of DNA. Serial dilutions of DNA were immobilized onto nitrocellul se filters and hybridized with the probe as described in the next sections.

Immobilization of DNA onto nitrocellulose filters. DNA samples were denatured at room temperature in the presence of 100 mM NaOH. After neutralization with 80 mM NaH_2PO_4 and adjusting the concentration to 6 × sodium chloride, sodium citrate solution (SSC) (1 × SSC: 150 mM NaCl, 15 mM sodium citrate), 200 μg total DNA were immobilized per each 2.5 cm diameter, 0.45 μm pore size nitrocellulose filter (Millipore Corp., Bedford, MA). The filters were dried under an infrared lamp, baked under vacuum at 80°C for 2 h, presoaked for 4 h at 42°C in a mixture containing 5 × SSC, 1 × Denhardt's solution [11], 0.1 mg ml^{-1} denatured salmon testes DNA (Sigma, St. Louis, MO), 0.2 mg ml^{-1} RNA (from Torula yeast, type VI, Sigma, St. Louis, MO), 20 mM HEPES (Sigma, St. Louis, MO) and 50% formamide.

Hybridization assay. Hybridization with ^{32}P-labeled p263-1 (approximately 3 × 10^6 cpm per filter) was carried out at 42°C for 15 h. The filters were washed for 5 min at room temperature with 2 × SSC, 0.5% SDS; 15 min with 2 × SSC, 0.1% SDS; followed by four washes at 54°C, 30 min each, with 0.4 × SSC, 0.1% SDS. After drying, the bound radioactivity was determined by scintillation counting.

Results

Standardization of the assay. The probe was tested in hybridization assays against various amounts of purified parasite DNA (Fig. 1). The lower sensitivity limit of the assay was 100 pg of parasite DNA, an amount approximately equivalent to 1000 haploid nuclei [12]. A linear relationship was obtained between the radioactivity detected and the amount of parasite DNA immobilized per filter. This standard curve was used to calculate the amount of parasite DNA present in the liver or spleen.

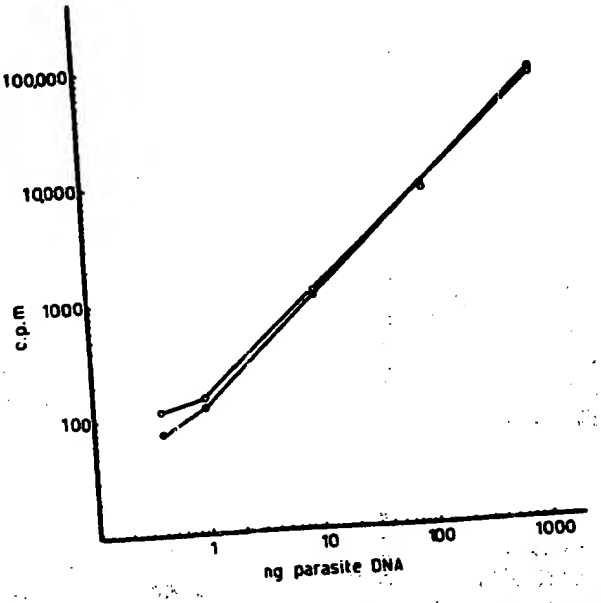

Fig. 1. Standard hybridization curves relating the amount of *P. berghei* DNA immobilized per nitrocellulose filter and the radioactivity bound. Open and black circles represent the results of two different experiments. Each value represents the mean of 3 filters. Standard deviations were negligible.

Since the DNA in a parasite nucleus is approximately 1% of that present in an hepatocyte nucleus, and the great majority of liver cells do not contain parasites, the ratio between host and parasite DNA will be very large, even at the peak of parasite proliferation. The possibility that the massive amounts of host DNA might interfere with the hybridization process was tested in a reconstruction experiment in which mixtures containing different ratios of parasite and host DNA were probed. As shown in Fig. 2, the presence of up to 25 000 times more liver DNA than parasite DNA did not have a significant effect on the assay.

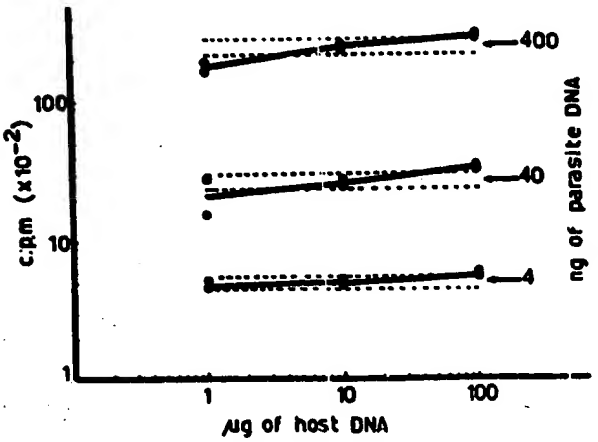

Fig. 2. Effect of host DNA on the hybridization assay. Different ratios of *P. berghei* DNA and normal rat liver DNA were immobilized onto nitrocellulose filters. A *P. berghei* DNA probe was labeled with ^{32}P by nick translation and hybridized with the immobilized DNA. Results are shown in the solid lines. The dotted lines show the range of results of hybridization assays performed in the absence of rat DNA.

Time course of parasite DNA proliferation. Because of its exquisite sensitivity to the hepatic stages of *P. berghei* [3–5], the Norway Brown rat was chosen as an animal model. Five groups of 3 rats each were inoculated i.v. with 3.4×10^5 sporozoites per animal. Livers and spleens were removed at 25, 30, 35, 44 and 54 h post-infection. DNA was purified, immobilized on nitrocellulose filters and hybridized with ^{32}P-labeled p263-1. The amount of parasite DNA detected per liver were 94, 850, 2500, 9000 and 7000 ng, respectively (Fig. 3). The peak of parasite DNA proliferation in the liver was at 44 h (9 µg parasite DNA or approximately 9×10^7 parasite nuclei). A small spleen signal was detected at 54 h. This is possibly due to contamination with peripheral blood or trapping of merozoites recently released from the liver.

When 10^6 sporozoites were injected per mouse, only 1.5×10^2 ng of parasite DNA (approximately 1.5×10^6 parasites) were detected per liver at 36 h (Fig. 4). In a different experiment (not included in Fig. 4), when 1.5×10^5 sporozoites were injected per mouse, 180 ng of *P. berghei* DNA (approximately 1.8×10^6 parasite nuclei) were detected at 41 h post infection. Since at 45 h there is already a sharp decrease in the hepatic parasite DNA content (Fig. 4), it is likely that the 41 h

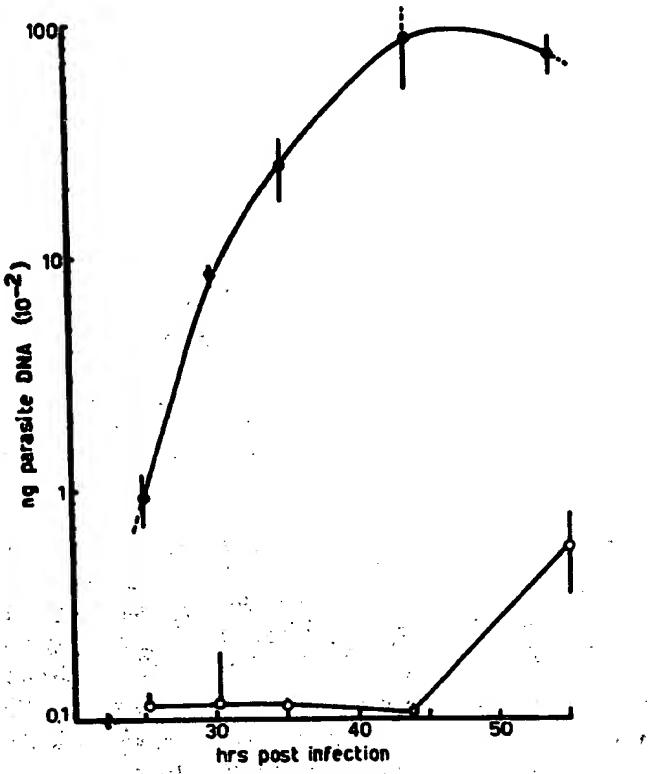

Fig. 3. Time-course development of *P. berghei* EEF in Norway Brown rats. 3.4×10^5 sporozoites were injected per rat. Parasite DNA per liver (●) and spleen (○) was calculated from 3 organs. Bars represent the standard deviations. These values were calculated by interpolating the amount of radioactivity detected per experimental filter (200 µg total liver DNA) into the standard curve (Fig. 1). The amount of parasite DNA thus obtained was corrected according to the total amount of DNA present in the individual organ.

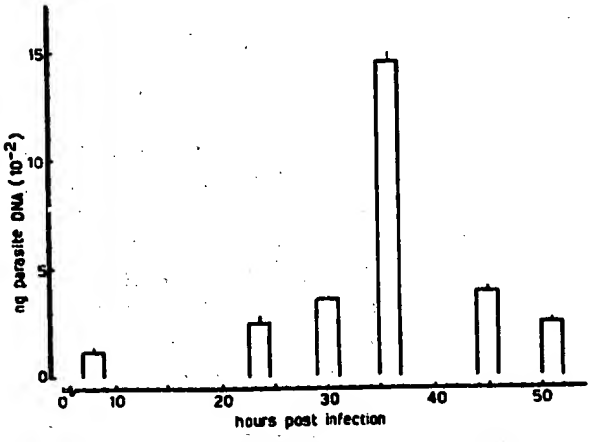

Fig. 4. Time-course of the development of *P. berghei* EEF in the livers of A/J mice. 10^5 sporozoites were injected per mouse. Values represent the mean number of parasite DNA content in the livers of two mice (calculated as in legend of Fig. 3).

value represents the true peak of *P. berghei* EEF proliferation in the mouse. If the amounts of parasite DNA in the rat (Fig. 3) and the mouse livers are compared at the peak of EEF proliferation, *P. berghei* sporozoites appear to be about 20 times more infective for rats. This is in agreement with prior observations [13] that A/J mice, although very sensitive to blood-induced infection, seem to poorly support the development of EEF.

Relationship between the number of sporozoites injected and the amount of parasite DNA per liver. Four groups of 3 rats each were injected with 10000, 40000, 1.6×10^5 and 6.4×10^5 sporozoites i.v. At 44 h post-infection, the amounts of parasite DNA per liver were 680, 3200, 6000 and 9600 ng. Thus, the proportion of sporozoites that successfully proliferate to EEF decreases with doses larger than 40 000. In agreement with the results shown in Fig. 3, no parasite DNA was detected in the spleens at this time (Fig. 5).

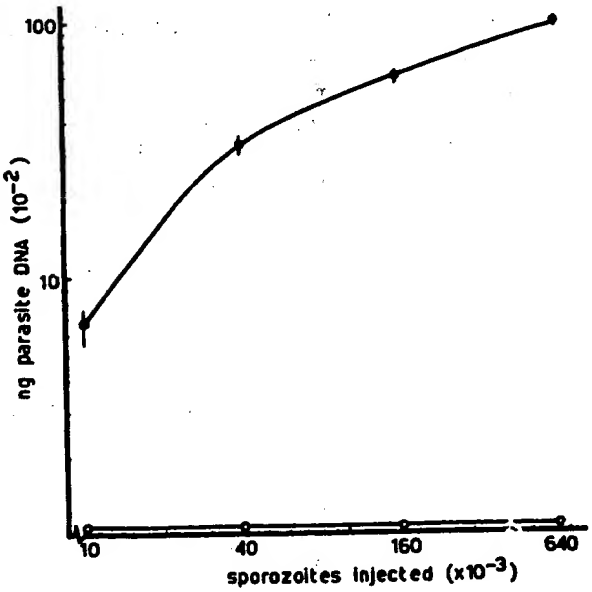

Fig. 5. Dose response experiment relating the number of sporozoites injected per Norway Brown rat with the amount of parasite DNA per liver (●) or per spleen (○) at 44 h post infection. Each value (calculated as in legend of Fig. 3) is the mean of results in three animals. Bars represent the standard deviations.

Effect of age on the amount of parasite DNA found at 44 h post-infection. Two groups of 4 rats each, aged 43 and 152 days and weighing, on average, 77 ±14 g and 186 ±20 g, respectively, were injected with 2.3×10^5 sporozoites i.v. and the livers probed at 44 h. No statistical difference ($P > 0.8$, calculated by a 2-tailed *t*-test) was found between the amounts of parasite DNA present in the livers of both groups (40.6 ± 27.3 ng and 44.2 ±5.4 ng, respectively).

Role of the spleen in the removal of sporozoites from circulation. Three rats were injected i.v. with sporozoites and, 5 h post-infection, spleens and livers were removed and probed. To permit the detection of parasite DNA at this early stage, when few if any cycles of replication have occurred, relatively large numbers of sporozoites (1.2×10^6 per rat) were injected. More parasite DNA was found in the spleen than in the liver (Table I, experiment 1) which contained only 7.5% of the injected parasites. To verify whether the spleen removes viable parasites from circulation, rats were splenectomized or sham operated and 10 days later injected intraportally with 5×10^4 sporozoites. Livers were probed 44 h post-infection. Approximately 8–9 times more parasite DNA was found in association with the livers of splenectomized animals (Table I, experiment 2). In a separate experiment (Table I, experiment 3) the effects of i.v. injections of 2.3×10^5 sporozoites in the portal and tail veins were compared. No significant difference ($0.8 > P > 0.6$) in the amounts of parasite DNA present in the liver were found. These results suggest that the spleen removes large numbers of infective sporozoites from circulation.

Discussion

Here we present an alternative method for the evaluation of sporozoite infectivity and development of EEF, based on the use of a specific DNA probe to measure the amount of parasite DNA in the liver of the host.

This method permits the detection of 100 pg of parasite DNA (Fig. 1), or 1000 haploid nuclei, assuming that each nucleus contains 0.1 pg of DNA [12]. Since 200 μg of DNA can be immo-

TABLE I

Role of spleen in the development of EEF of *P. berghei* sporozoites in Norway Brown rats

Experiment[a]	Treatment	Sporozoites injected ($\times 10^5$)	Route of injection	Organ probed	Time of organ removal (hours post-infection)	ng parasite DNA per organ ($\times 10^2$)	P^b
1	–	12	i.v. tail	spleen	5	1.18 ± 0.9	0.05–0.025
				liver		0.09 ± 0.02	
2	splenectomy	0.5	portal	liver	44	174.6 ± 16.6	<0.0005
	sham					20.5 ± 1.8	
3	laparotomy	2.3	portal	liver	44	22.8 ± 11	0.06–0.8
			i.v. tail			28.2 ± 19	

[a] Experiments done on different dates with different batches of sporozoites and nick translation reagents.
[b] Calculated by 2-tailed *t* tests.

bilized per nitrocellulose filter and, according to our measurements, rat liver contains 12.5 ± 1.63 mg of DNA ($n = 50$), the minimum number of parasites detectable per liver is about 62 500. This represents the progeny of only 6–7 sporozoites at the peak of their proliferation, since each can generate close to 10^4 haploid nuclei after 13 nuclear divisions [14]. Therefore this assay is ideally suited to measure the effect of antisporozoite agents and vaccines. For example, when 4×10^4 sporozoites are injected into rats, 3.2×10^7 haploid nuclei are generated at 44 h (Fig. 5). Assuming that about 7.5% of the inoculum enter liver cells (Refs. 6,15 and Table I, experiment 1) this represents the progeny of only 4×10^3 sporozoites. If the penetration and/or proliferation of the infective sporozoites were to be inhibited by 99%, the remaining 40 sporozoites would have a progeny of 4×10^5 haploid nuclei or 700 pg parasite DNA per filter, a value still within the sensitivity limits of the assay. The choice of the rat rather than the mouse as an animal model greatly contributed to the increase in sensitivity of the assay. Both young and old rats can be used since, as stated in the Results section, there is no difference in the amount of parasite DNA found in their livers following injection of identical numbers of sporozoites. The observed resistance of older rats [13] to infection with *P. berghei* sporozoites is probably caused by post-hepatic events in the parasite's cycle. For example, young rats infected with *P. berghei* have large numbers of circulating reticulocytes, a preferred target of these parasites.

The efficiency of the rat liver to capture sporozoites and support EEF development seems to be much higher than that of the mouse (Fig. 3 and 4). The precise reasons for this are not clear, since at 8 h post infection approximately 10% of the sporozoites injected into mice are found in association with the liver (Fig. 4). It is unlikely that a sizable portion of the parasite DNA in the mouse liver at 8 h represents sporozoites cleared by Kupffer cells, since experiments recently performed in our laboratory (U. Vergara et al., personal communication) demonstrate that close to 100% of the liver-associated parasite DNA is found in the hepatocytic fraction. This fraction was separated from the Kupffer cells in a Percoll gradient, 3 h after *P. berghei* sporozoite infection. Alternatively, the proportion of sporozoites undergoing full EEF development may be much larger in rat than in mouse hepatocytes. The latter hypothesis is supported by the observation that at 25 h post-infection, close to 100 ng or 10^6 *P. berghei* haploid nuclei per rat liver were detected (Fig. 3), when 3.4×10^5 sporozoites were injected. Assuming that 7.5% entered and developed in hepatocytes [6,14,15], approximately 5 mitotic cycles would have occured at this time. In contrast, in the mouse most of the DNA proliferation occurred only 30 h after injection (Fig. 4).

The release of mature merozoites from the EEF is a rather swift process. Time course experi-

ments (Fig. 3) indicate that the maximum EEF number was found approximately 44 h post-infection, while by 70 h no parasite DNA was detectable in the liver (results not shown). This finding is in agreement with previous histological studies [5,16].

Interestingly, the amount of parasite DNA in the liver at 44 h did not increase linearly with the number of parasites injected. With an inoculum >40 000 sporozoites, the curve flattened (Fig. 5). This was unexpected in view of the large excess of hepatocytes available as targets for parasite penetration. Further studies are underway to determine whether with an increasing inoculum size fewer parasites enter hepatocytes or whether the apparent restrictions for development occur within the target cells.

In malaria it is well known that the spleen removes infected red blood cells from circulation. Our findings show that at 5 h post-infection, a large number of sporozoites is found in the spleen (Table I, experiment 1) and that, following splenectomy, the number of EEF in the liver increases several-fold (Table I, experiment 2). The simplest explanation for these observations is that the spleen is also an important barrier and trap for infective sporozoites, but the precise mechanisms for their recognition are unknown.

In short, although laborious, the method of quantitating *P. berghei* DNA described here should permit not only sensitive, specific measurements of sporozoite infectivity, and the evaluation of the effects of drugs, antibodies, etc. on the parasite, but also tracing their distribution and development inside the mammalian host.

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Differentiation of Toxoplasma gondii from closely related coccidia by riboprint analysis and a surface antigen gene polymerase chain reaction.
Brindley P J; Gazzinelli R T; Denkers E Y; Davis S W; Dubey J P; Belfort R; Martins M C; Silveira C; Jamra L; Waters A P; et al
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DIFFERENTIATION OF *TOXOPLASMA GONDII* FROM CLOSELY RELATED COCCIDIA BY RIBOPRINT ANALYSIS AND A SURFACE ANTIGEN GENE POLYMERASE CHAIN REACTION

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Abstract. The tachyzoite of the human pathogen *Toxoplasma gondii* is morphologically indistinguishable from the proliferative stages of some other zoonotic coccidia, including *Sarcocystis*. To determine the identity of such coccidia obtained from human tissues and other sources, we compared riboprints (through restriction enzyme analysis of the polymerase chain reaction [PCR]–amplified small subunit rRNA gene) of the following protozoa: the RH and ts-4 strains of *T. gondii*, lines OH3 and S11, which are two recently isolated *T. gondii*–like parasites from Brazil, *Neospora caninum*, *Sarcocystis* species, and the malarial parasite *Plasmodium berghei*. In addition, the protozoan genomes were examined by PCR for homologs of surface antigen genes of *T. gondii*, and by Southern hybridization to the heterologous rRNA gene probe pSM 389. Strains OH3, S11, ts-4, and RH shared identical riboprints, and OH3, S11, and ts-4 have p22 and p30 surface antigen gene structures similar to RH. In contrast, riboprints for *N. caninum* and *T. gondii* differ with respect to *Dde* I sites, and moreover, their genomes vary significantly from one another at both the p22 and p30 gene loci. The riboprints of *Sarcocystis* and *P. berghei* differ markedly from *T. gondii* and *N. caninum* and from each other. *Bam* HI pSM 389 restriction fragment length polymorphisms differentiate ts-4 from RH, OH3, and S11. Our results confirm that OH3 and S11 are indeed *T. gondii*, but that *N. caninum* and *T. gondii* are likely to be separate species, thereby resolving previous uncertainties concerning the identity of these parasites. Together, the variation in riboprints and surface antigen gene structure reflects the phylogenetic diversity among these coccidia, and in addition, confirms the value of riboprinting in the identification of apicomplexan parasites such as *T. gondii*.

Human populations are constantly exposed to and infected by *Toxoplasma gondii*, and it is estimated that toxoplasmosis exists in a chronic asymptomatic form in 20–30% of the people in the United States.[1] Whereas infection with *T. gondii* is usually innocuous or asymptomatic, it causes serious morbidity or mortality in the developing fetus and in other immunocompromised individuals.[2,3] Moreover, concurrent infection with *T. gondii* is of increasing concern in the context of acquired immunodeficiency syndrome (AIDS). Indeed, it is reported that diagnosis of toxoplasmosis leads, in turn, to the diagnosis of approximately 5% of new cases of AIDS.[4]

Notwithstanding the serologic and symptomologic methods for diagnosis of toxoplasmosis,[5] the differential identification of *T. gondii* from related apicomplexan protozoa is not straightforward because there exists extreme morphologic similarity among the proliferative stages of these coccidia,[6–8] and because the parasites are promiscuous in host range.[8] In particular, the tachyzoite of *T. gondii* can probably infect any nucleate mammalian or avian cell and other coccidia such as *Sarcocystis* and *Hammondia* share similarly broad host ranges, including humans and other primates.[8–11]

Recently, polymerase chain reaction (PCR) techniques have been adapted to the issue of diagnosis of human infection with *T. gondii*. Burg and others have used a PCR specific for the B1 gene of *T. gondii*,[11] and PCR detection of the p30 surface gene[12] has been recently shown to detect *T. gondii* infection in pathologic specimens of human eye tissues embedded in paraffin.[13] In a similar fashion, PCR-based techniques have been used to identify other protozoa. Using riboprint-

ing, which involves restriction endonuclease analysis comparisons of the PCR-amplified small subunit ribosomal RNA (SSU rRNA) gene, Clark and Diamond demonstrated variability in the ribosomal genes of *Entamoeba* species, and showed that some *E. histolytica*–like amoebae, including the Laredo strain, are in fact strains of *E. moshkovskii*, and not are closely related to *E. histolytica*.[14]

Because of this success, and in view of the results using the PCR to diagnose toxoplasmosis described above, we have applied riboprinting to the differentiation of closely related apicomplexan parasites, including species of zoonotic coccidia indistinguishable by light microscopy from the human pathogen *T. gondii*. The coccidia examined included protozoa of human and porcine origin from Brazil, where an enigmatic focus of ocular toxoplasmosis has been recently reported.[15] In addition to riboprinting, analysis by PCR for the presence of genes encoding two surface antigens of *T. gondii* was used. Our results demonstrate that these procedures can distinguish *T. gondii* from other morphologically inseparable coccidia, including *Neospora caninum*[16] and *Sarcocystis* spp., which can parasitize the same hosts.

MATERIALS AND METHODS

Parasites and extraction of genomic DNA

Th following protozoa and mammalian cells were examined in this study: tachyzoites of the RH and ts-4 strains of *T. gondii*;[17] tachyzoites of the OH3 and S11 lines of *T. gondii*–like parasites that were isolated in 1989 in the town of Erexim, Rio Grande do Sul, Brazil from the eye of a human patient with unilateral retinochoroiditis (OH3),[18] and from pork sausage intended for human consumption (S11);[19] tachyzoites of *N. caninum*, a *T. gondii*–like parasite of cattle, dogs, and other mammals, and for which the life cycle and source of infection are not yet known;[7, 20] bradyzoites of three species of the cyst-forming coccidia *S. cruzi*, *S. suicanis*, and *S. hominis*;[11] merozoites of a malarial parasite, *Plasmodium berghei*; and murine, bovine, and human cells in which the protozoan lines were maintained. The RH and ts-4 strains were maintained in human foreskin fibroblasts at 37°C and 33°C, respectively; strains OH3 and S11 were isolated from experimentally infected mice by peritoneal lavage; *P. berghei* was isolated from blood of infected mice; and *N. caninum* was maintained in vitro in bovine monocytes.[20] Bradyzoites of *Sarcocystis* were obtained by pepsin digestion of intramuscular sarcocysts from experimentally infected cattle.[9]

Genomic DNAs were obtained by standard methods. Briefly, cells were suspended in 50 mM Tris, pH 8, 100 mM NaCl, 25 mM sucrose, 10 mM EDTA, 100 μg/ml of proteinase K (Gibco-BRL, Gaithersburg, MD), and 1% sodium dodecyl sulfate (SDS), and incubated for 4 hr at 37°C with end-over-end agitation. A one-tenth volume of 2 M KCl was added and the mixture was incubated for 15 min at room temperature to precipitate the SDS and proteins, which were subsequently removed by centrifugation. The DNA was extracted from the supernatant by partitioning against phenol/chloroform, recovered by precipitation with ethanol in the presence of sodium acetate, and its purity was verified by its optical density at a wavelength of 280 nm.

Polymerase chain reaction

The PCR[21] was performed using a kit (GeneAmp; Perkin-Elmer-Cetus, Norwalk, CT) and a thermal cycler (MJ Research, Watertown, MA). Each PCR proceeded through 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Oligonucleotide primers were obtained from Synthecell Corp. (Rockville, MD). Primers P5 (5′-GGAAGCTTATCTGGTTGATCCTGCCAGTA-3′) and P3 (5′-GGGATCCTGATCCTTCCGCAGGTTCACCTAC-3′), which flank the SSU rRNA gene of *Entamoeba histolytica*,[14] and which are similar to primers described by Medlin and others,[22] were used to amplify the SSU rRNA gene from genomic DNAs of coccidian parasites by the PCR. These primers are designed to hybridize to highly conserved sequences at the extreme 5′ (P5) and 3′ (P3) termini of eukaryotic SSU rRNAs. Thus, the PCR amplifies a fragment that is essentially the full sequence of the SSU-rRNA gene.[14, 22] In addition, other primers were used to amplify ~500 basepair (bp)– and ~1,000-bp fragments, respectively, of the p22 and p30 surface antigen genes of the tachyzoite stage of the RH strain of *T. gondii*.[12, 23] These primers are p22, 5′: 5′-AACAGAAGATCTAAAATGAGTTTCTCAAAGAACACGAGC-3′; p22, 3′: 5′-GGGCTACACAAACGTGATCAACAAACCTGCGAGACC-3′; p30, 5′: 5′-AACAGAAG-

Restriction enzymes used in
*zoa**

Enzyme	Se
Alu I	AG
Hha I	GC
Rsa I	GT
Mbo I	†G
Tha I	CG
Mae II	A†
Dde I	C†
Msp I	C†
Taq I	T†
Hae III	GG
Mse I	T†
Nla I	CA
Rma I	C†

* Target recognition sequences for
were RH, ts-4 (for strain RH and ts-4
(Ne), *Plasmodium berghei* (Pb), and S
The Southern hybridization column i
SSU rRNA gene of RH was perform

ATCTATGTCGGTTTC
A-3′; p30, 3′: 5′-GGGTC
GCTGCGATAGAGCC-

Riboprinting

The riboprint analysis
scribed previously for *E*
the present analyses use
zymes. Aliquots of each
after electrophoresis thr
in Tris/borate/EDTA (T
89 mM boric acid, 2 mM
with DNA standards (
products were purified f
primers by chromatogra
ose spin columns (5′-3′,
by digestion with indivi
ucleases for 4 hr under
conditions recommende
of the enzymes. Appro
product was digested w
zyme. The following 13
have recognition sequen
and therefore cut DNA
generate comparative r
parasites: *Alu* I, *Hha* I, *R*
I, *Tha* I, *Hae* III, *Dde* I
Mse I, *Rma* I, and *Nla* III
Biolabs, Beverly, MA), a

TABLE 1

*Restriction enzymes used in the riboprint analysis of the small subunit (SSU) rRNA genes of coccidian protozoa**

Enzyme	Target sequence	Parasites	Southern hybridization
Alu I	AG†CT	RH, OH3, S11, ts-4, Nc, Pb	−
Hha I	GCG†C	RH, OH3, S11, ts-4, Nc, Pb	−
Rsa I	GT†AC	RH, OH3, S11, ts-4, Nc, Pb	−
Mbo I	†GATC	RH, OH3, S11, ts-4, Nc, Pb, Sc	+
Tha I	CG†CG	RH, OH3, S11, ts-4, Nc, Pb, Sc	+
Mae II	A†CGT	RH, OH3, S11, ts-4, Nc, Pb, Sc	+
Dde I	C†TNAG	RH, OH3, S11, ts-4, Nc, Pb, Sc	+
Msp I	C†CGG	RH, OH3, S11, Nc, Pb	+
Taq I	T†CGA	RH, OH3, S11, Nc, Pb	+
Hae III	GG†CC	RH, OH3, S11, Nc	−
Mse I	T†TAA	RH, OH3, S11, Nc	−
Nla I	CATG†	RH, OH3, S11, Nc	−
Rma I	C†TAG	RH, OH3, S11, Nc	+

* Target recognition sequences for the enzymes are given, with a dagger at the cleavage point. The parasites from which the genes were amplified were RH, ts-4 (for strain RH and ts-4 of *Toxoplasma gondii*), OH3, and S11 (for two Brazilian isolates of *T. gondii*–like parasites), *Neospora caninum* (Nc), *Plasmodium berghei* (Pb), and *Sarcocystis cruzi* (Sc). Following digestion, the restriction fragments were separated by agarose gel electrophoresis. The Southern hybridization column indicates whether or not hybridization of these fragments to the radiolabeled polymerase chain reaction–amplified SSU rRNA gene of RH was performed.

ATCTATGTCGGTTTCGCTGCACCACTTC A-3'; p30, 3': 5'-GGGTCACGCGACACACAA GCTGCGATAGAGCC-3'.

Riboprinting

The riboprint analysis was performed as described previously for *E. histolytica*,[14] although the present analyses used other restriction enzymes. Aliquots of each PCR product were sized after electrophoresis through 0.8% agarose gels in Tris/borate/EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) by comparison with DNA standards (Gibco-BRL). The PCR products were purified from the oligonucleotide primers by chromatography using G-50 sepharose spin columns (5'-3', Malvern, PA), followed by digestion with individual restriction endonucleases for 4 hr under buffer and temperature conditions recommended by the manufacturers of the enzymes. Approximately 1 μg of PCR product was digested with 10–20 units of enzyme. The following 13 enzymes, all of which have recognition sequences of four nucleotides and therefore cut DNA frequently, were used to generate comparative riboprints for coccidian parasites: *Alu* I, *Hha* I, *Rsa* I, *Mbo* I, *Msp* I, *Taq* I, *Tha* I, *Hae* III, *Dde* I (all from Gibco-BRL), *Mse* I, *Rma* I, and *Nla* III, (all from New England Biolabs, Beverly, MA), and *Mae* II (Boehringer Mannheim, Indianapolis, IN). The fragments generated by digestion with these enzymes were separated through 2.6% agarose gels (3:1 NuSieve:Seakem LE; FMC Bioproducts, Rockland, ME) in TBE buffer, stained with ethidium bromide, and sized by comparison with standards (phage λ + *Hind* III, phage φX174 RF + *Hae* III, and/or a 1-kb ladder (Gibco-BRL).

In some experiments, the fragments were transferred by Southern blotting to nylon membranes (GeneScreen Plus; New England Nuclear, Wilmington, MA) by capillarity. The PCR-amplified SSU rRNA gene of RH strain of *T. gondii* was radiolabeled with ^{32}P-dCTP (Amersham, Arlington Heights, IL)) by nick translation[24] and used to probe the Southern blots, using hybridization conditions as described by Brindley and others[25] and stringent washing in 0.1 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate) at 65°C for 1 hr. Autoradiography was performed at −70°C with intensifying screens. Table 1 lists the restriction enzymes (and their recognition sequences) used with each species, and the South rn hybridizations performed.

The p22 and p30 surface antigen genes

The p22 and p30 genes of *T. gondii* encode 22-kD[23] and 30-kD[12] surface antigens, respectively, on the tachyzoite. Oligonucleotide prim-

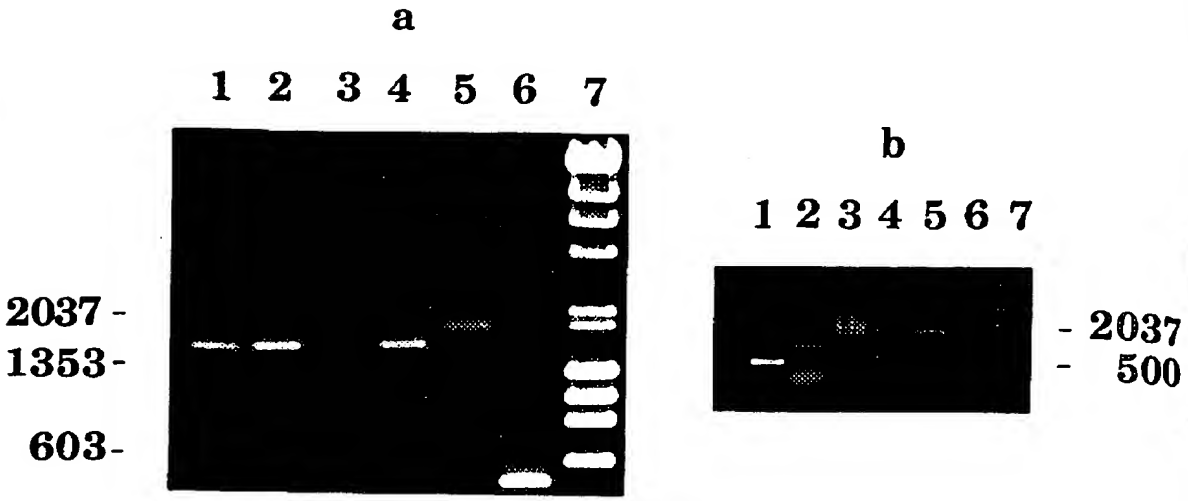


FIGURE 1. Polymerase chain reaction (PCR) analyses of genomic DNAs from coccidian protozoan parasites and mammalian host cells using the primers specific for eukaryotic small subunit rRNA genes. Molecular sizes are given at the margins in nucleotide basepairs. **a**, target DNA templates. Lane 1, *Toxoplasma gondii* RH strain; lane 2, OH3; lane 3, S11; lane 4, *Neospora caninum;* lane 5, *Plasmodium berghei;* lane 6, phage lambda DNA (control for the PCR); lane 7, DNA size standards (λ + *Hind* III and φX174 RF + *Hae* III). **b**, target DNA templates. Lane 1, lambda (PCR control); lane 2, *Sarcocystis hominis;* lane 3, *S. suicanis;* lane 4, *S. cruzi;* lane 5, *T. gondii* ts-4; lane 6, bovine macrophages; lane 7, DNA size standards (λ + *Hind* III).

ers (above) specific for both of these genes of the RH strain of *T. gondii*[12, 23] were used in PCR analyses to detect the presence of these genes in the other coccidia examined here.

Hybridization to pSM 389

To further investigate genomic variation among the Brazilian isolates OH3 and S11 versus the RH and ts-4 lines of *T. gondii*, ^{32}P-labeled pSM 389, a plasmid probe that contains part of the SSU rRNA gene plus some nontranscribed spacer sequence from the rDNA of *Schistosoma mansoni*[25] was hybridized to a Southern blot of *Bam* H1-digested (Gibco-BRL) genomic DNAs from RH, ts-4, S11, and OH3. The rationale for using prob pSM 389 was that while the coding region for rRNA would be expected to be evolutionarily conserved, mutations might be expected to have accumulated in the flanking regions that separate the multiple copies of the rRNA genes, and that these changes might be detectable with probe pSM 389. (A heterologous probe was used because cloned fragments from the ribosomal gene unit of *T. gondii* have not been reported.)

RESULTS

PCR amplification of SSU rRNA genes

The rDNA primers supported the PCR amplification of the SSU rRNA gene from all protozoan DNAs examined, but not from the mammalian DNA templates under the same conditions (Figure 1) The PCR products amplified from RH, S11, OH3, ts-4, and *N. caninum* were each approximately 1,950 nucleotides (nt) in length. Only a single product was amplified. In contrast, PCR of *Sarcocystis* DNAs yielded a product of approximately 1,950 nt, but there were other, less prominent fragments amplified in each of the three species. In *P. berghei*, a single product of approximately 2,100 nt was amplified, which is the expected size for the SSU rRNA gene in this malarial species.[26]

Riboprint analysis

The *T. gondii* RH and ts-4 strains and the *T. gondii*–like isolates OH3 and S11 from Brazil shared identical riboprints. Based on the patterns of the ethidium bromide–stained fragments generated with each of the 13 frequently cutting enzymes and on Southern hybridization analysis of

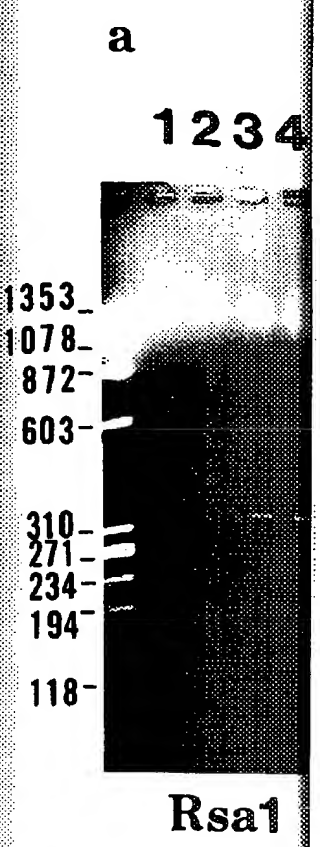


FIGURE 2. Representativ
reaction (PCR)–amplified s
coccidia. **a**, SSU rRNA gene
I, *Hha* I, or *Alu* I. **b**, SSU
Neospora caninum (lane 4) d
are shown at the margins of

these fragments using the r
gene of RH as the prob
evident among these fou
tative results with the enz
I, *Mbo* I, and *Dde* I are s
3a and Table 1.

The riboprint for the *N*
gene was similar to those
S11 with 12 of the 13 enz
ever, *Dde* I digestion of
RH generated eight frag
620, 350, 290, 240, 180
whereas digestion of the
duced seven fragments (a
325, 290, 240, 80 and 60
hybridization analysis of
probed with the radiola
from RH indicated that

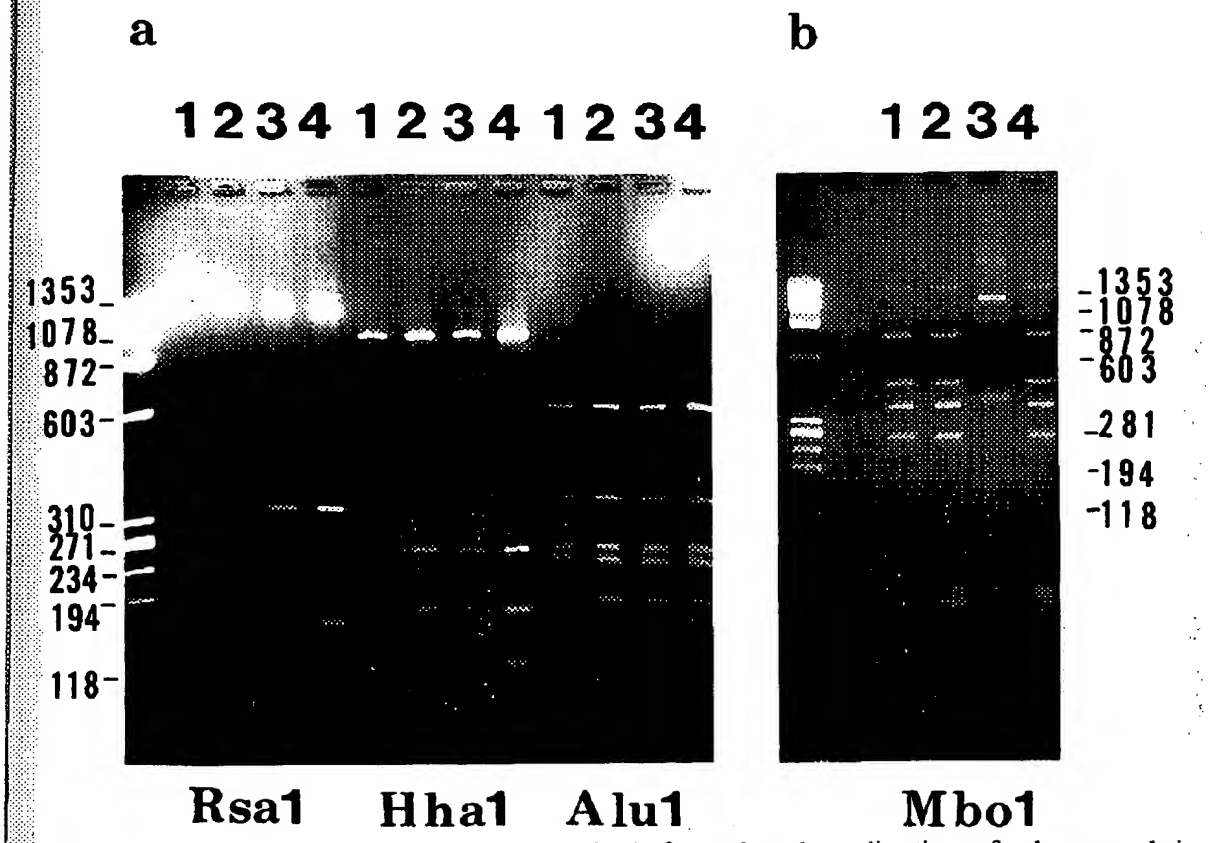


FIGURE 2. Representative riboprint patterns obtained after endonuclease digestions of polymerase chain reaction (PCR)–amplified small subunit (SSU) rRNA genes from strains of *Toxoplasma gondii* and related coccidia. **a**, SSU rRNA genes from OH3 (lane 1), RH (lane 2), S11 (lane 3), and ts-4 (lane 4) digested with *Rsa* I, *Hha* I, or *Alu* I. **b**, SSU rRNA genes from OH3 (lane 1), RH (lane 2), *Plasmodium berghei* (lane 3), and *Neospora caninum* (lane 4) digested with *Mbo* I. DNA standards in nucleotide basepairs (ϕX174 RF + *Hae* III) are shown at the margins of the gels.

these fragments using the radiolabeled SSU rRNA gene of RH as the probe, no differences were evident among these four parasites. Representative results with the enzymes *Rsa* I, *Hha* I, *Alu* I, *Mbo* I, and *Dde* I are shown in Figures 2 and 3a and Table 1.

The riboprint for the *N. caninum* SSU rRNA gene was similar to those of RH, ts-4, OH3, and S11 with 12 of the 13 enzymes (Figure 2b). However, *Dde* I digestion of the SSU rRNA gene of RH generated eight fragments (approximately 620, 350, 290, 240, 180, 145, 80, and 60 nt), whereas digestion of the *N. caninum* gene produced seven fragments (approximately 620, 350, 325, 290, 240, 80 and 60 nt) (Figure 3a). Southern hybridization analysis of the blotted *Dde* I digests probed with the radiolabeled SSU rRNA gene from RH indicated that all the *Dde* I fragments of both species' genes are indeed from the PCR-amplified rDNA and not PCR artifacts (Figure 3b). Thus, *N. caninum* exhibits a similar riboprint to *T. gondii*, but lacks one *Dde* I site present in the latter.

The riboprint for *S. cruzi* differed from that of *T. gondii* (and *N. caninum*) for each of the four enzymes used for these particular comparisons (*Dde* I [Figure 3b], *Mbo* I, *Tha* I, and *Mae* II [Table 1]). Since differences were evident with each of the four enzymes used, and given that the recognition sequences of these four enzymes are dissimilar (Table 1), substantial evolutionary divergence appears to exist between the SSU rRNA sequences of *Sarcocystis* and *Toxoplasma*. In like fashion, the riboprints for *P. berghei* differed with each of the nine enzymes used (Table 1) from those of *T. gondii* and from *N. caninum*

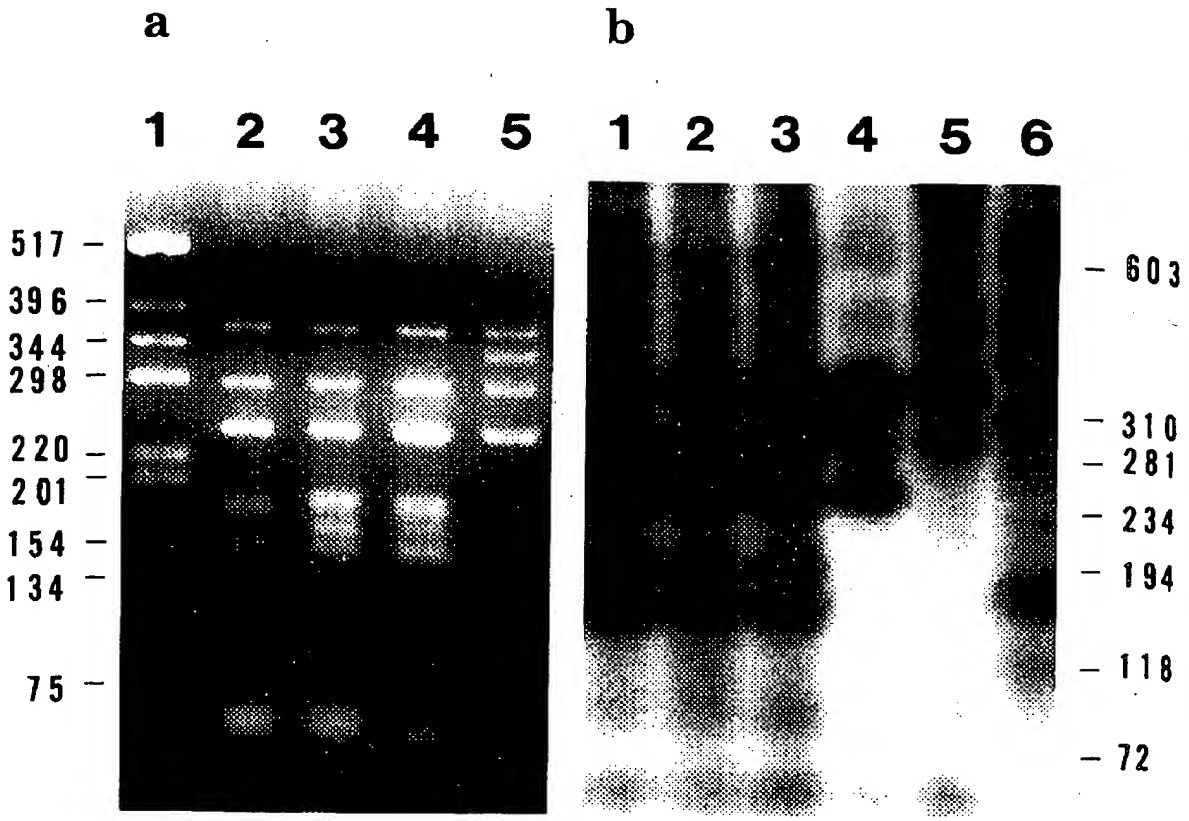


FIGURE 3. **a**, ethidium bromide–stained fragments after *Dde* I digestion of polymerase chain reaction (PCR)–amplified small subunit (SSU) rRNA genes from *Toxoplasma gondii* RH (lane 2), OH3 (lane 3), S11 (lane 4), and *Neospora caninum* (lane 5). Lane 1 contains molecular size standards in basepairs (1-kb ladder). **b**, Southern hybridization of radiolabeled PCR-amplified SSU rRNA gene from strain RH to *Dde* I-digested genomic DNAs from *T. gondii* ts-4 (lane 1), OH3 (lane 2), S11 (lane 3), *N. caninum* (lane 4), *Sarcocystis cruzi* (lane 5), and *Plasmodium berghei* (lane 6). The hybridization patterns to ts-4, OH3, and S11 are identical, whereas *N. caninum* differs in having an extra hybridization band at 325 bp, and no hybridization bands at 180 bp and 145 bp. The hybridization patterns for *S. cruzi* and *P. berghei* show increasing dissimilarity from the other parasites. Size standards in basepairs (ϕX174 RF + *Hae* III) are shown at the right margin.

(e.g., *Mbo* I, Figure 2b and *Dde* I, Figure 3b). Moreover, *P. berghei* differed from *S. cruzi* for each of the four restriction enzymes used to compare these two species (e.g., *Dde* I, Figure 3b).

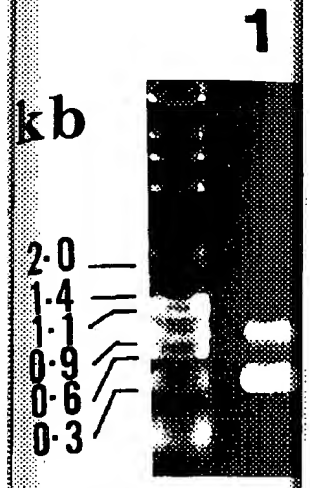
Since there are, on average, five target recognition sequences in the SSU rRNA gene of these coccidia for each restriction enzyme used, and given that the SSU rRNA gene is approximately 1.95 kb in length, the riboprints analyzed approximately 13% of the nucleotide sequence of the SSU rRNA gene of each coccidian.

Surface antigen gene PCR

The diagnostic primers for both the p22 and p30 genes supported the amplification of target sequences of approximately 500 nt and 1,000 nt, respectively, from template DNA from the RH strain of *T. gondii*, from both the Brazilian OH3 and S11 *T. gondii*–like protozoa (Figure 4), and from ts-4. In contrast, no products of the expected 500 nt and 1000 nt were amplified from *N. caninum* or *S. cruzi* templates (Figure 4), or from the *P. berghei* or mammalian (bovine and murine) DNA.

Southern hybridization of genomic fragments to pSM 389

Southern hybridization analysis of *Bam* H1-digested genomic DNA with radiolabeled pSM 389 gave the same pattern for RH, OH3, and S11, but a different pattern for ts-4. In particular, ts-4 exhibits hybridization signals at 11 kb and



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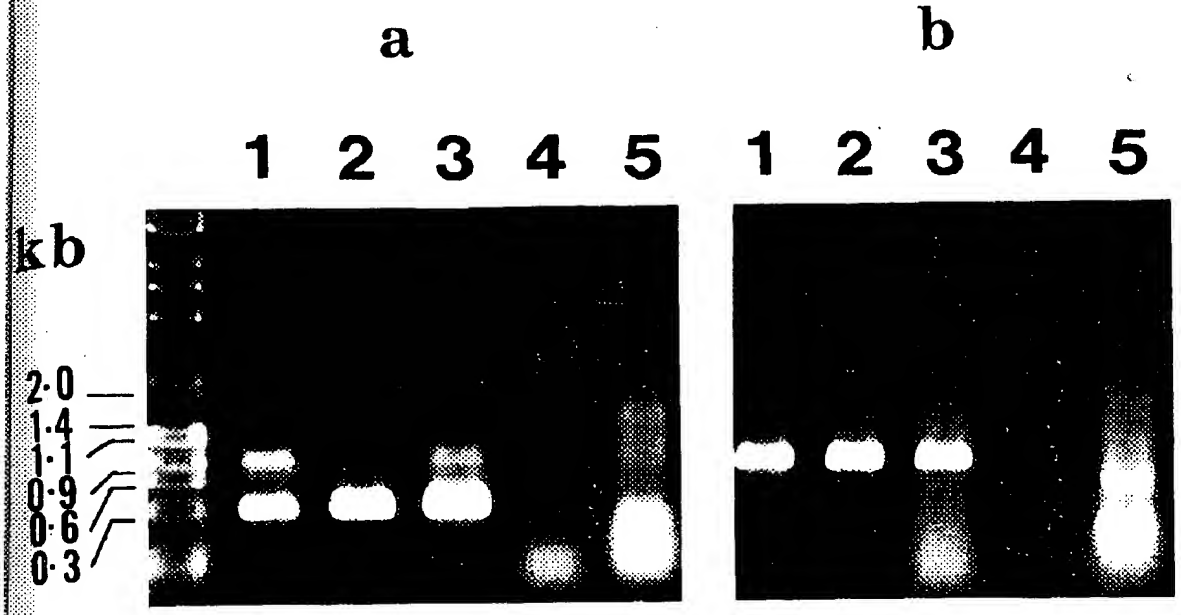

FIGURE 4. Surface antigen gene polymerase chain reaction (PCR) analyses of genomic DNAs from coccidian parasites. Template DNAs from *Toxoplasma gondii* RH (lane 1), OH3 (lane 2), and S11 (lane 3) *Neospora caninum* (lane 4), and *Sarcocystis cruzi* (lane 5) were primed with oligonucleotides specific for the p22 (**a**) or the p30 (**b**) surface antigen genes of *T. gondii*.[12, 23] Control PCRs using the same templates with the rDNA primers gave products of the expected sizes. DNA size standards in kilobasepairs (kb) are shown in the leftmost lane of gel **a**.

5.5 kb, which are approximately 500 bp larger in each case than corresponding signals from RH, OH3, and S11. In addition, the ts-4 lane shows a band of hybridization at 2 kb, which is absent from the others (Figure 5).

DISCUSSION

Oligonucleotide primers related to those described by Medlin and others[22] supported PCR amplification of the SSU rRNA genes of *T. gondii* and related coccidia, but not of mammals. Based on the sequences of the PCR primers, the amplified product can be expected to include the complete SSU rRNA gene sequence and nothing else.[14, 22] The SSU rRNA gene is of a similar length in RH, ts-4, OH3, S11, *N. caninum*, and *Sarcocystis*, whereas the gene in *P. berghei* is substantially larger (2.1 kb versus 1.95 kb).[26] However, determination of the size of the gene in the *Sarcocystis* spp. was less precise, since several smaller fragments were amplified in the PCR, in addition to a product of approximately 2 kb. The extra bands in *Sarcocystis* most likely result from sequences other than the SSU rRNA genes, which have sufficient similarity to the primers to allow annealing in the PCR. Alternatively, the patterns with *Sarcocystis* may indicate that one or more of the tandemly arrayed rRNA genes in these parasites is truncated, resulting in the PCR amplification of fragments of several sizes. If so, the putative truncation mutations vary among the *Sarcocystis* species.

The OH3, S11, ts-4, and RH strains of *T. gondii* shared identical riboprints. In contrast, riboprints for *N. caninum* and *T. gondii* differed with respect to a *Dde* I site. The riboprints of *S. cruzi* and *P. berghei* differ markedly from those of *T. gondii* and *N. caninum* and from each other. Because the present riboprint results demonstrate directly that differences exist in the ribosomal gene sequences of these coccidia, it may be feasible to design and use diagnostic *T. gondii*–specific oligonucleotide probes based on the SSU rRNA gene sequence. Indeed, it has been shown recently that specific oligonucleotide probes based on SSU rRNA gene sequences can differentiate among all four species of malarial parasites that are pathogenic in humans.[27]

One aim of the present study was to determine the genetic relationship of coccidia isolated recently in Brazil to laboratory strains of *T. gondii*.

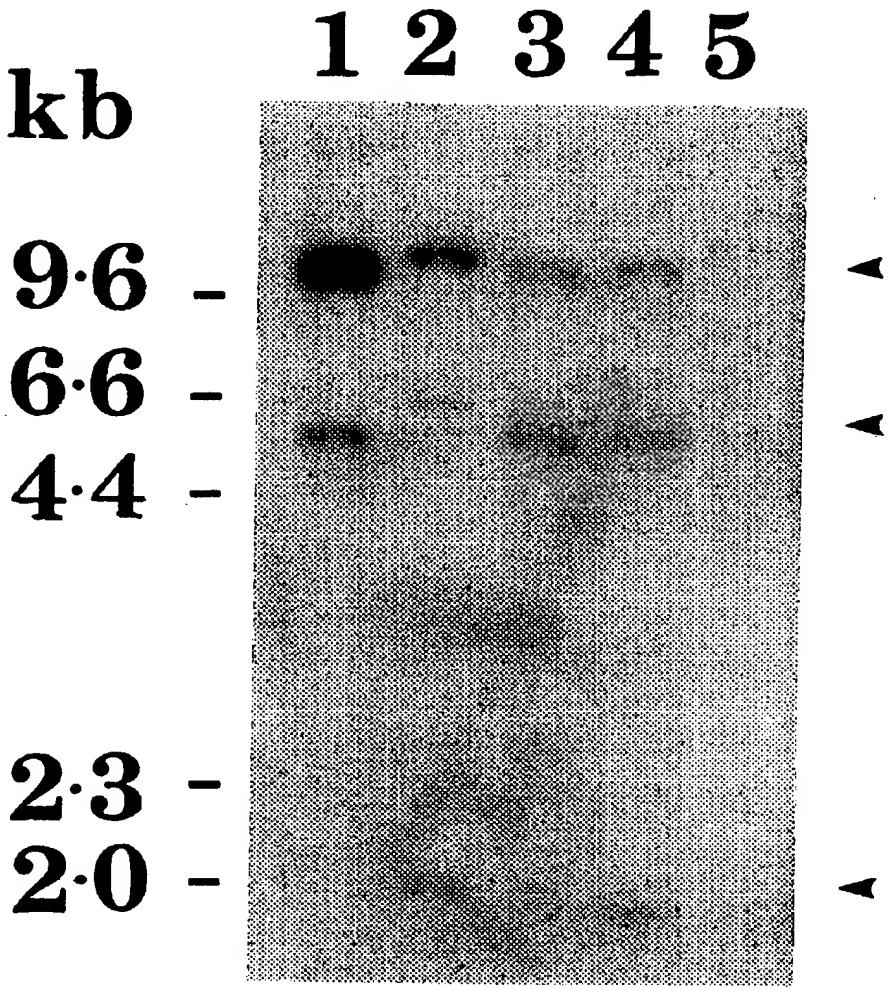

FIGURE 5. Southern hybridization of radiolabeled probe pSM 389 to *Bam* H1-digested genomic DNAs from *Toxoplasma gondii* RH (lane 1), ts-4 (lane 2), OH3 (lane 3), S11 (lane 4), and mouse (lane 5). The hybridization pattern to the ts-4 genome differs (**arrowheads**) from that for RH, OH3, and S11, which are identical to each other. No hybridization is evident to the murine DNA. DNA size standards in kilobasepairs (kb) (λ + *Hind* III) are shown at the left.

The OH3 and S11 *Toxoplasma*–like parasites were isolated at Erexim, Rio Grande do Sul State where an unusual transmission of toxoplasmosis-induced retinochoroiditis has been reported;[15] it involves familial infection of more than one (non-twinned) sibling. Such a pattern is not consistent with congenital transmission since asymptomatic mothers previously exposed to *T. gondii* are classically assumed to be immune to reinfection, and therefore, congenital infection is not expected in more than one pregnancy.[1] Alternatively, the ocular toxoplasmosis at Erexim may be acquired postnatally and involve a pathogenic form of *T. gondii* that can cause serious disease in previously exposed individuals. Nevertheless, riboprints for the *Toxoplasma*–like isolates OH3 and S11 are indistinguishable from the RH strain of *T. gondii*, which together with the presence of the p22 and p30 surface antigen gene sequences in OH3 and S11, indicate that these Brazilian isolates are indeed forms of *T. gondii*, despite the unusual epidemiology apparent at Erexim.

In addition to the riboprint and surface antigen gene comparisons of RH of the Brazilian isolates, we compared several biological characteristics between OH3 and S11 and virulent (RH) and avirulent (C56[11]) strains of *T. gondii*. First, OH3 and S11 exhibit similar virulence to RH in BALB/c and C57Bl/6 mice with respect to the

ethal dose of tachyzoites. Second, they showed decreased in vivo sensitivity to sulfadiazine compared with RH (Gazzinelli RT, and others, unpublished data). These biological similarities provide further indications that OH3 and S11 are virulent forms of *T. gondii*.

Whereas the riboprints of RH, ts-4, OH3, and S11 indicate strongly that they have very similar or identical SSU rRNA genes, the Southern hybridization pattern of radiolabeled pSM 389 to the genomic DNAs from these parasites indicate some differences in rDNA between ts-4 and RH, but not among OH3, S11, and RH. Given the identical riboprints, it appears that the *Bam* H1/pSM 389 restriction fragment length polymorphisms (RFLPs), which differentiate *T. gondii* strain ts-4 from RH, have arisen through mutations in the spacer sequences that separate the copies of the rRNA-encoding DNA, rather than in the SSU rRNA gene sequences. Genomic differences between ts-4 and RH are not surprising since ts-4 was derived through chemical mutagenesis of RH and is temperature sensitive, unlike RH.[17] In a similar fashion, the RFLPs that distinguish ts-4 from RH may have arisen during the same mutagenesis episode responsible for the temperature sensitivity of ts-4.

The riboprints, surface antigen gene analyses, and Southern hybridizations demonstrate a close but increasingly dissimilar genetic relationship among the *T. gondii* isolates and other coccidia analyzed; RH, OH3, S11 appear identical, ts-4 is slightly divergent, there is further divergence to *N. caninum*, and the greatest genetic distance is to *Sarcocystis* and *P. berghei*. With respect to *N. caninum*, its variant SSU rRNA and p22 and p30 surface antigen gene structure support previous epidemiologic, serologic, and ultrastructural evidence indicating that *N. caninum* and *T. gondii* are distinct pathogens.[7] Moreover, the recent results of Burg and others,[11] which demonstrate the absence of the *T. gondii* B1 gene from the genome of *N. caninum*, as determined by B1-specific PCR analysis, provide a further difference between the two protozoa.

Finally, approximately 13% of the nucleotide sequences of the coccidian SSU rRNA genes were examined using 13 restriction enzymes in our riboprint analysis. The riboprint technique has the advantage that it is faster than determining the full 2-kb nucleotide sequence of the SSU rRNA genes of each organism being compared. In addition, since the riboprints for OH3 and S11 strongly suggest that these Brazilian isolates are ind ed *T. gondii*, the procedure appears to be of value for diagnosing species of apicomplexa. Thus, the present results support the findings of Clark and Diamond[14] and Rowan and Powers[28] that have demonstrated the utility of the technique in taxonomic studies with other morphologically indistinguishable taxa, and they expand the range of target microbes bearing SSU rRNA genes that can be amplified with the primers of Medlin and others.[22]

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sity seems to go slightly down and then shifts within minutes to a density that is higher than the one observed in their vegetative phase (Fig. 3). This process takes less than 30 min and is the first noticeable response during encystation, long before any morphological or biochemical changes can be detected. The reasons for this process and the mechanism by which it is evoked are a subject for further study; however, if *E. invadens* IP-1 encystation is indeed a good model for the encystation process of other *Entamoeba,* this phenomenon may serve as a test to see if other media may induce the eventual encystation of *E. histolytica.* Preliminary results that suggest osmotic pressure may also be a trigger for the encystation of *E. histolytica* were recently obtained in our laboratory. Transfer of xenic cultures of *E. histolytica* SAW 408 from a medium of high osmotic pressure to one with lower osmotic pressure afforded small (5%), but reproducible, yields of cysts.

In contrast, our attempts to induce encystation in either the axenic *E. invadens* 165 strain or the *E. histolytica* 200:NIH or in 200:NIH grown in NRS flora were unsuccessful under any of the conditions described. Therefore, the question as to whether all *Entamoeba* strains have the capacity to encyst (in laboratory cultures) is still open. Different conditions for different strains may be required in order to promote their encystation. Alternatively, it is possible that some strains that have been kept for long periods of time under laboratory conditions in vitro have become altered and may no longer be capable of undergoing encystation.

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Sequence of the Small Subunit Ribosomal RNA Gene Expressed in the Bloodstream Stages of *Plasmodium berghei*: Evolutionary Implications[1]

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ABSTRACT. We have determined the complete nucleotide sequence of the coding region of the small subunit rRNA gene expressed by bloodstream stages of the apicomplexan *Plasmodium berghei.* It is 2059 nucleotides long. Elements contributing to its relatively large size are all concentrated in regions known to be variable in length among eukaryotes. In a phylogenetic tree constructed from pairwise comparisons of eukaryotic small subunit rRNA sequences, the apicomplexan line branches at a rather early point in eukaryotic evolution before any multicellular kingdoms had yet appeared.

THE evolutionary origins of the apicomplexans remain obscure in spite of our constantly increasing knowledge of their biology and morphology. During their adaptation to parasitism, these organisms may have undergone considerable phenotypic change. A flagellate ancestry for the apicomplexans has been suggested because these two groups share several common life cycle features, including zygotic meiosis and flagellated gametes (28). The oyster parasite *Perkinsus marinus* and the small predatory flagellate *Spiromonas perforans* also serve as potential connections between the two groups. *Perkinsus marinus* is placed within the apicomplexans on the basis of zoospore ultrastructure

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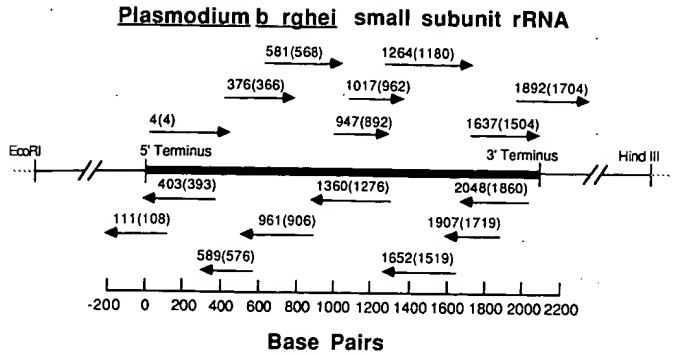

Fig. 1. Restriction map and sequencing strategy of the *P. berghei* small subunit rRNA gene. The region coding for the small subunit rRNA lies within a 5.6 kb *Eco*RI/*Hin*d III restriction fragment. Synthetic DNA oligomers (Table I) complementary to evolutionarily conserved regions on both coding and noncoding strands were used as primers in dideoxynucleotide chain-termination sequencing reactions. The arrows indicate both the extent and direction of the sequence read from a particular primer site. The locations of the primers in the *P. berghei* rRNA gene are indicated above the arrows; the numbers in parentheses represent their positions in the *Dictyostelium discoideum* small subunit rRNA.

(conoid, polar ring, micropores, and rhoptries) and yet its zoospores have flagella (21). *Spiromonas perforans* shares many morphological features with the apicomplexans including micronema, micropores, mitochondria with tubular cristae, a three-membrane pellicle, and as is the case with apicomplexans, it penetrates other cells (1). To what extent these shared features represent convergence rather than common ancestry is unknown. Therefore, it is difficult to establish unequivocally the relationships of apicomplexans to other protists.

Features of morphology and life cycle used by protozoologists to determine relationships may have been altered the most during adaptation to intracellular parasitism. Other cellular components which share common functional roles in all organisms are less likely to undergo radical change. Comparisons drawn from evolutionary markers less directly involved in adaptations to particular ecological niches may reveal the relationships of apicomplexans to other protists. It is likely that the molecular mechanisms for transcription and translation are quite similar in apicomplexans and their ancestors. Sequence information from macromolecules involved in these processes can be used to determine the extent of genetic relatedness between organisms. Ribosomal RNAs, particularly the small subunit rRNAs (SSU rRNAs), are considered to be among the more useful macromolecules for phylogenetic studies (8, 31, 34). These molecules are functionally conserved in all known organisms and can be used to measure both distant and close evolutionary relationships. We have taken advantage of the highly conserved sequences that are distributed throughout the gene to determine rapidly the nucleotide sequence of the SSU rRNA coding region within the ribosomal RNA cistron that is expressed in the bloodstream forms of *Plasmodium berghei*. In a phylogeny constructed from comparisons of SSU rRNAs, *P. berghei* is seen to diverge from the eukaryotic line of descent significantly later than did the euglenoids or kinetoplastids but prior to the appearance of numerous other eukaryotic microorganisms including ciliates, the acanthamoebae, and the fungi.

MATERIALS AND METHODS

Construction of the subclone pBbS5.6 containing the SSU rRNA coding region from the Unit-A rRNA cistron (which is expressed in bloodstream stages of *P. berghei* strain NUY2) has been previously described (4). An *Eco*RI/*Hin*d III restriction fragment containing the SSU rRNA coding region was separated from plasmid DNA by agarose gel electrophoresis (13) and extracted from the gel by the freeze-thaw method (29). The fragment was then recloned into the replicative form (RF) of the single stranded M13mp18 and M13mp19 phages (17, 20) propagated in *Escherichia coli* strain JM109 (36).

Our use of the primer extension/dideoxynucleotide chain termination protocols (26) for rapidly sequencing eukaryotic SSU rRNA's has been previously described in greater detail (6). Single-stranded M13 templates which include the coding and noncoding regions for the SSU rRNA's are annealed to synthetic DNA oligonucleotide primers (prepared as in 15) that are complementary to evolutionarily conserved regions. The sequence and locations of primers in eukaryotic rRNA's are given in Table I and the sequencing strategy is illustrated in Fig. 1. From a given primer it is possible to determine the sequence of 400–600 positions. For our analysis of the *P. berghei* SSU rRNA we have changed the annealing solution (for placing the synthetic primers onto the single-stranded templates) to 25 mM Tris-HCl, (pH 7.2), 25 mM $MgCl_2$, 2 mM dithiothreitol and we have altered the dideoxynucleotide concentrations as follows: ddA, 0.088 mM; ddG, 0.34 mM; ddT, 0.5 mM; ddC, 0.5 mM; and ddG/deoxyinosine (dI) to 0.175 mM/0.2 mM in order to increase the length of sequence which can be read from each sequencing reaction. The reactions were terminated by the addition of EDTA and sodium acetate to final concentrations of 10 mM and 100 mM, respectively, and the primer extension products were recovered by precipitation with 2.5 volumes of ethanol. The precipitates were washed in 80% ethanol, dried under vacuum, and then resuspended in 12.5 μl of gel-loading buffer (0.1% xylene cyanol and 0.1% bromphenol blue in formamide). Two-microliter samples of each sequencing reaction mixture were electrophoresed on 40 cm 6% or 8% polyacrylamide sequencing gels (25) as previously described (6). Sequence determinations beyond 250 positions were ascertained from

TABLE I. *Synthetic DNA oligonucleotides used as primers in determining the sequence of the* Plasmodium berghei *small subunit rRNA coding region.*

Location in *P. berghei*	Location in *D. discoideum*	Sequence[a]
4–20	4–20	C T GGTTGATCCTGCCA G[b]
376–392	366–382	A G GGTTCGATTCCGGA G[b]
581–596	568–583	C GGTAATTCCAGCTCC[b]
947–961	892–906	Y A GAGGTGAAATTCT[b]
1017–1031	962–976	A T CAAGAACGAAAGT[b]
1264–1279	1180–1195	T T TGACTCAACACGGG[b]
1637–1652	1504–1519	C AGGTCTGTGATGCT C[b]
1892–1908	1704–1720	T GYACACACCGCCCGT C[b]
111–97	108–94	C T GTTTTAATGAGCC[c]
403–387	393–377	T CAGGCTCCCTCTCC G G[c]
589–572	576–559	G WATTACCGCGGCKG C T G[c]
961–947	906–892	A GAATTTCACCTCT G[c]
1360–1346	1276–1262	C GGCCATGCACCACC[c]
1652–1637	1519–1504	G GGCATCACAGACCT G[c]
1907–1893	1719–1705	A C GGGCGGTGTGTR C[c]
2048–2033	1860–1845	C Y GCAGGTTCACCTA C[c]

[a] The symbols used here are those recommended by the IUB nomenclature committee; K represents G or T; R represents G or A; W represents A or T; and Y represents T or C.

[b] Synthetic DNA oligonucleotides complemenary to the coding strand of the small subunit rRNA gene.

[c] Synthetic DNA oligonucleotides complementary to the non-coding strand of the small subunit rRNA gene.

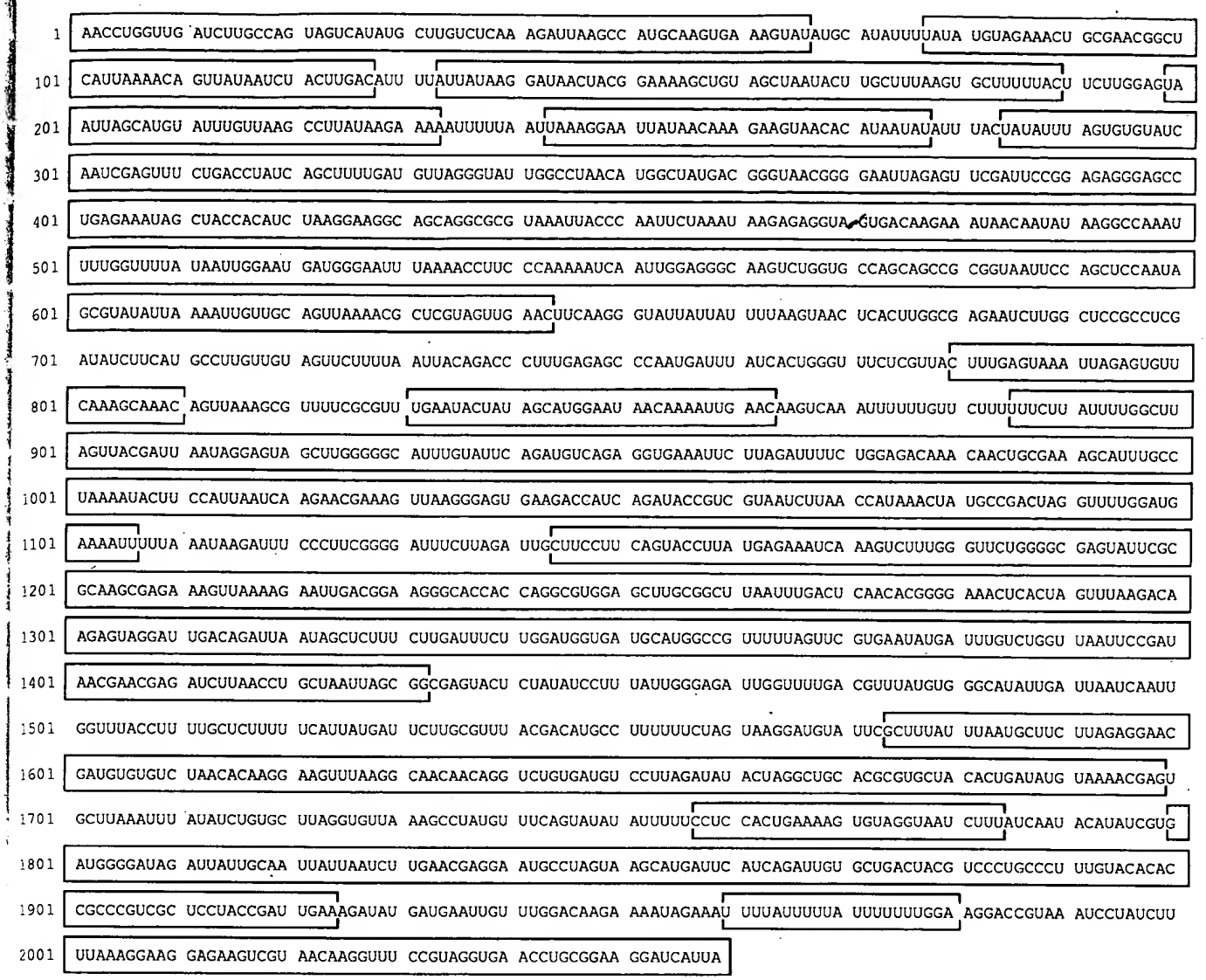

Fig. 2. Sequence of the small subunit rRNA coding region expressed in bloodstream stages of *P. berghei*. The boxes define those positions which can be unambiguously aligned in comparisons of the eukaryotic small subunit rRNAs.

nongradient 80-cm gels built in 1 × NNB (134 mM Tris base, 45 mM boric acid, and 2.5 mM EDTA).

Alignment of the SSU rRNA sequences with each other has been described elsewhere (6). The similarity values generated by all possible pairwise comparisons of sequences were used in the formula of Jukes & Cantor (11) to estimate the number of evolutionary changes separating each pair of the sequences. These values ("structural distance" values) were used to produce phylogenetic trees by a variation of the method of Fitch & Margoliash (7). In brief, trees were generated in which the branch lengths separating each pair of organisms approximated as closely as possible the structural distance values calculated as separating each pair of organisms. The methods for evaluating alternative tree topologies have been published elsewhere (6, 10, 12).

RESULTS

Eukaryotic SSU rRNAs are typically 1850–1900 nucleotides in length but they can range in size from 1753 nucleotides in *Tetrahymena thermophila* (33) to 2305 nucleotides in *Euglena gracilis* (31). The coding region for the *Plasmodium berghei* SSU rRNA presented in Fig. 2 is 2059 nucleotides long, and the extra nucleotides (relative to typical eukaryotic SSU rRNAs) occur within regions that are known to be highly variable in length and sequence composition (9). The only unusual length variation in the *P. berghei* sequence lies within the helix defined by positions 1418 to 1597. This stem and loop structure is 133 nucleotides longer than the homologous structure in *T. thermophila*. The only other eukaryote that is known to extend significantly the length of this structure (to 154 nucleotides, 107 nucleotides longer than in *T. thermophila*) is *A. castellanii*.

A consideration of highly conserved positions in eukaryotic SSU rRNAs can be used to assess evolutionary relationships. Certain positions are invariant or nearly so in all published eukaryotic SSU rRNAs. Some indication of the depth of branching for a lineage leading to a particular organism is given by the number of positions in its SSU rRNA which differ from an rRNA consensus sequence of the other eukaryotes. There are a total of 617 positions which are invariant in sequence comparisons of rat (2), mouse (22), *Xenopus laevis* (24), *Artemia salina* (19), *Saccharomyces cerevisiae* (14, 23), rice (35), *Zea*

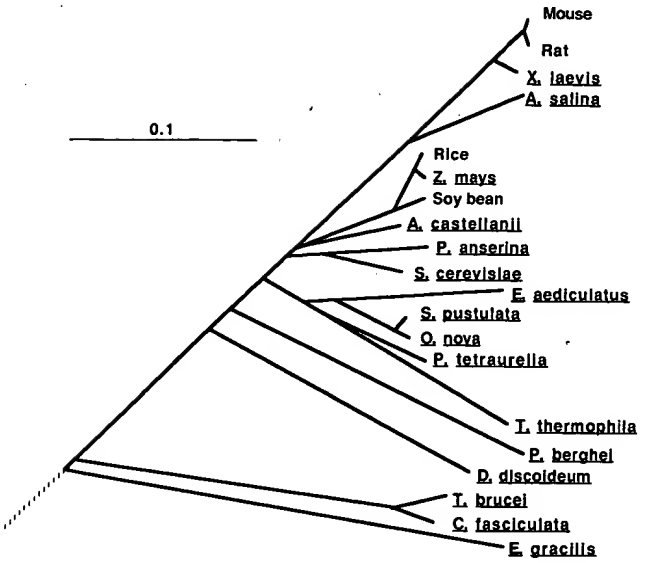

Fig. 3. Phylogeny inferred from small subunit rRNA sequence similarities. A phylogenetic tree was inferred by using the structural distances separating each pair of sequences. The analysis is based on a comparison of 1663 positions. The evolutionary distance between organisms is represented by the horizontal components of their separation in the figure.

mays (18), soy bean (5), *Crithidia fasciculata* (27), *Acanthamoeba castellanii* (9), *Dictyostelium discoideum* (16), *Tetrahymena thermophila* (33), *Paramecium tetraurelia* (30), *Euplotes aediculatus* (32), *Stylonychia pustulata* and *Oxytricha nova* (6), *Euglena gracilis* and *Trypanosoma brucei* (31), *Podospora anserina* (Sogin, unpubl. data), and *P. berghei*. When *P. berghei* is excluded from such an analysis, there are 638 invariant positions; thus *P. berghei* differs from the consensus sequence of the other eukaryotic SSU rRNA sequences listed above at a total of 21 positions. In a similar analysis for protists that represent early branchings in the eukaryotic phylogenetic tree (31), *D. discoideum* differs from other eukaryotes at 19 positions; the kinetoplastids (as represented by *T. brucei* and *C. fasciculata*) differ at 60 positions, and *E. gracilis* violates 70 conserved positions in the eukaryotic consensus sequence. These departures from the eukaryotic consensus sequences can be interpreted as representing the relative depths of branching of the organisms considered. The divergence of *P. berghei* would appear to correspond to a relatively early period in eukaryotic evolution during which the evolutionary lineage leading to the cellular slime molds (as represented by *D. discoideum*) also appeared.

This estimation of divergence patterns is supported by a more detailed analysis in which structural distances are converted into phylogenetic trees using the distance matrix methods described above. Structural similarities and distances must be computed from positions which can be unequivocally aligned in all of the compared sequences. Higher resolutions in such comparisons can sometimes be achieved by increasing the number of positions considered (and therefore increasing the amount of evolutionary information); however, there is an inverse correlation between the number of positions which can be compared and the phylogenetic distance between the sequences being compared. In addition to point mutations, deletions and insertions accumulate as sequences drift apart from each other. The resulting length variations between SSU rRNAs makes it difficult and in some cases impossible to align unambiguously all positions. For example, when prokaryotic SSU rRNAs are included in a structural similarity calculation for the eukaryotes, approximately 689 positions defining primary and secondary structures which are distinctive to eukaryotic SSU rRNAs must be excluded. We have used the largest number of positions possible by limiting the analysis to eukaryotes where 1663 nucleotide positions can be unambiguously aligned. The structural similarities and structural distances between the SSU rRNA of *P. berghei* and those of other eukaryotes are not presented in order to conserve space, but are available on request from the authors. Structural similarity values range from 0.646 (between *Euglena gracilis* and *P. berghei* sequences) to 0.799 (between *Oxytricha nova* and *P. berghei* sequences). These values were computed using the nucleotide positions that are enclosed within the boxes in the *P. berghei* SSU rRNA sequence displayed in Fig. 2.

The distance matrix tree derived from this data is presented in Fig. 3. The tree is "unrooted" in that it does not include a prokaryotic representative which could serve as an "out group." In a multi-kingdom analysis that included representatives from both the eubacterial and archaebacterial lines of descent (31), *E. gracilis* and *T. brucei* were found to represent the earliest eukaryotic branchings yet characterized by using comparisons of SSU rRNA sequences. We have assigned the root of the phylogenetic tree in Fig. 3 on the basis of that analysis. This phylogeny shows that the evolutionary lineage leading to *P. berghei* diverged from other eukaryotes slightly after the cellular slime molds appeared and long after euglenoids and kinetoplastids arose. The divergence of the apicomplexan lineage clearly predated the appearance of ciliates, acanthamoebae, and the three multicellular kingdoms.

DISCUSSION

The distance matrix tree in Fig. 3 indicates that the protistan lineage leading to apicomplexans diverged from other eukaryotes at a rather early point in their evolutionary history, before the appearance of any of the three multicellular kingdoms or the ciliates. Since no metazoans existed at the time when the apicomplexan ancestors diverged from the other protists, they must have had a long history as free-living organisms before their adaptation to parasitism. The phylogenetic tree indicates the general evolutionary position of the apicomplexans in relation to some other protistan groups but also shows that the apicomplexan line is not closely related to any of the other branches in the tree. This may be due to the small number of organisms represented in the tree or to the actual absence of closely related forms among extant organisms. If *S. perforans* is actually an aberrant kinetoplastid, it is not likely to represent a link to the apicomplexans (as represented by *P. berghei*). Our analysis suggests a very distant relationship between kinetoplastids and apicomplexans. The phylogenetic affinities of *S. perforans* remain obscure although it has some similarities to dinoflagellates (1) and it would be useful to compare its SSU rRNA sequence to the rRNAs of other protists. The closeness of *P. marinus* to the apicomplexans is also controversial (3). Unfortunately, using rRNA sequence information to settle the problem is not practical since raising and collecting sufficient quantities of *P. marinus* is not possible at this time.

Major differences between sequences can be due to long and separate evolutionary histories between organisms or they may reflect an unusually rapid rate of evolutionary change in one of the sequences considered. Therefore, it is of interest to determine whether or not the relatively early divergence of the apicomplexan line is a reflection of a high mutation rate or "fast evolutionary clock" in its SSU rRNAs. If a sequence is evolving

rapidly, its homology to a distantly related sequence will be lower than the homology of a more slowly evolving sequence to the same distantly related sequence. An earlier analysis showed that *E. gracilis* and the kinetoplastids represent the earliest divergences among the eukaryotes (31). The structural similarity value between *P. berghei* and *E. gracilis* (0.646) can be compared with that between *D. discoideum* and *E. gracilis* (0.652); the values show that the apicomplexan lineage has a clock speed only slightly faster than that of the cellular slime mold lineage. Therefore, the depth of divergence must reflect time of divergence rather than unusual clock speed. Furthermore, the normal clock speed indicates that adaptation to parasitism has not affected the rate of change of the rRNA; the profound morphological response visible to a microscopist is not duplicated at other levels of cellular organization.

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Plastid Origin of an Extrachromosomal DNA Molecule from *Plasmodium*, the Causative Agent of Malaria

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Several species of *Plasmodium* have been shown to contain a circular extrachromosomal DNA molecule which is widely supposed to be mitochondrial DNA. However, it has recently been shown to have a number of features in common with chloroplast DNA. Here, a phylogenetic analysis of RNA polymerase coding sequences from the *Plasmodium* molecule has been carried out using distance matrix, maximum likelihood, parsimony and operator invariant methods. The analysis indicates that the molecule is in fact derived from an oxygenic photosynthetic organism and should be regarded as plastid DNA. This suggests that *Plasmodium* originated from a phototroph that has lost the capacity to photosynthesize.

Introduction

Several species of *Plasmodium*, the causative agent of malaria, have been shown to contain a conserved circular extrachromosomal DNA molecule of c. 35 kb.p. (Kilejian, 1975; Dore *et al.*, 1983; Williamson *et al.*, 1985; Gardner *et al.*, 1988). This has often been assumed to be mitochondrial DNA, since it is found in subcellular fractions that contain mitochondria (Kilejian *et al.*, 1975; Dore *et al.*, 1983). However, these fractions also contain several other organelles of ill-defined nature, and a number of mitochondrial gene sequences, such as those for cytochrome oxidase subunit *I* and apocytochrome *b*, have been shown to reside on a different extrachromosomal DNA molecule (Aldritt *et al.*, 1989; Vaidya *et al.*, 1989), so the assignment of the 35 kbp species to the mitochondrion should be regarded as tentative. The 35 kbp molecule of *P. falciparum* has been shown to contain genes for rRNAs (in an inverted repeated configuration) and subunits (*rpoB* and *rpoC*) of an RNA polymerase similar to that found in prokaryotes and chloroplasts (Gardner *et al.*, 1991). These features are also found in chloroplast DNA (Palmer, 1990) but they do not themselves provide direct evidence that the *Plasmodium* 35 kbp extrachromosomal molecule originated within a photosynthetic lineage. The possession of RNA polymerase genes would be an ancestral character (present both in photosynthetic and non-photosynthetic organisms and like, for example, the use of the "universal" genetic code), rather than shared and derived, and it would therefore not be phylogenetically informative. It is not known whether the possession of rRNA genes in an inverted repeated configuration is ancestral or derived. However, phylogenetic analysis of the sequence data should allow the origin of the 35 kbp circular molecule to be determined. Analysis of the rRNA sequences has been reported as providing "suggestive" evidence that it

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may have originated within a photosynthetic lineage (Gardner *et al.*, 1991). Here an analysis of the *rpoC* sequence that also indicates an origin within a photosynthetic lineage is described.

Methods

Nucleotide and deduced amino acid sequence data from the *rpoC* genes (*rpoC1* in the case of cyanobacteria and chloroplasts but referred to here as *rpoC*) of *Escherichia coli* (EM:ECRPOBC), *Nostoc commune* UTEX584 (EM:NCRPOCB) and *Anabaena* sp. (EM:ASRNAPOL) (cyanobacteria), and chloroplasts of *Euglena gracilis* (EM:CHEGRPO), liverwort (*Marchantia polymorpha*—EM:CHMPXX), spinach (*Spinacia oleracea*—EM:CHSORPOB) and tobacco (*Nicotiana tabacum*—EM:CHNTXX) were used for this purpose. Sequence manipulations and analysis were carried out using programs from the GCG, PHYLIP 3.2 and PHYLIP 3.4 packages mounted on the Cambridge University School of Biological Sciences VAX computer (Devereux *et al.*, 1984; Felsenstein, 1988, 1991).

Pairwise alignments were carried out using GAP with predicted amino acid sequences. Nucleotide sequences of only those regions that could be aligned unambiguously, without gaps and beginning and ending with amino acids conserved across all the species were used for the analysis (Fig. 1). Distance matrix analysis was carried out on 100 sequences bootstrapped from the original dataset using the program SEQBOOT. Distance matrices were calculated using DNADIST, the two-parameter model of Kimura and a transition/transversion ratio of 1·6 (Beanland, 1990). Trees were constructed using NEIGHBOR, implementing the neighbor-joining method of Nei and Saitou, and the consensus tree calculated with CONSENSE. Maximum Likelihood analysis was carried out using DNAML, with weightings corresponding to substitution rates of 2, 1 and 5 at the first, second and third codon positions respectively, the F and T options, and a transition/transversion ratio of 1·6 (Felsenstein, 1988; Beanland, 1990). Parsimony analysis was carried out using DNAPARS and the same weightings as for DNAML. Trees were tested using the U (user-defined tree) option in DNAML and DNAPARS and the site-by-site pairwise test of Kishino & Hasegawa (1989), which allows an estimate of whether or not a given tree is less likely or parsimonious at a given level of significance than the most likely or parsimonious one. Analysis using the operator invariant method was carried out using DNAINVAR.

Results and Discussion

Distance matrix analysis of bootstrapped data from *rpoC* generated the tree shown in Fig. 2(a). In all 100 of the bootstrapped trees constructed either using all three codon positions or positions 1 and 2 only, the *Plasmodium* sequence was placed in a clade with the plastids, and in 96 trees (omitting third position data, 89 when third position data were included) this was specifically with the *Euglena* sequence.

Maximum likelihood analysis using all eight taxa generated a best tree as shown in Fig. 2(b). As with the distance matrix analysis, the *Plasmodium* sequence was

```
C  RRERMGHIELASPTAH    PVLPPDLRPLVPLDGGRFATSDLNDLYRRVINRNN
A  RRHRMGYIKLAAPVAH    PVIPPDLRPMVQLDGGRFATSDLNDLYRRVINRNN
N  RRHRMGYIKLAAPVAH    PVIPPDLRPMVQLDGGRFATSDLNDLYRRVINRNN
E  RRFRMGYIDLVPPLIH    PVLPPDIRPVVKLQDGTVIMTDLNFLYIDIIYGNN
L  RRYRMGYIKLACSVTH    PVLPPELRPMIELGEGELITSDLNELYRRVIYRNN
T  RRYQMGYIKLACPVTH    PVLPPELRPIIQIDGGKLMSSDINELYRRVIYRNN
S  RRYQMGYIKLACPVTH    PVLPPELRPIIQIDGGKLMSSDINELYRRVIYRNN
P  RKYKLGFIFLNIPILH    PILPAGLRPYFYINNSTYIISTINENYRLIILKNN

C  GKQGRFRQNLLGKRVDYSGRSVITVGPYLRLHQCGLPKKMALEL
A  GKQGRFRQNLLGKRVDYSGRSVIVVGPKLKIHQCGLPREMAIEL
N  GKQGRFRQNLLGKRVDYSGRSVIVVGPKLKIHQCGLPREMAIEL
E  GKKGRFRENLLGKTVDYSGRSVIVVEPKLLLHECGMPLDIALEL
L  GKEGRFRENLLGKRVDYSGRSVIVVGPFLPLHQCGLPREMAIEL
T  GKEGRFRETLLGKRVDYSGRSVIVVGPSLSLHRCGLPREIAIEL
S  GKEGRFRETLLGKRVDYSGRSVIVVGPSLSLHRCGLPREIAIEL
P  GKYSTIKYKLLGKRVDFSGRSVITVNPSIIYNNIGLPYYISINL

C  NRAPTLHRLGIQAFEPVLIEGKAIQLHPLVCAAYNADFDGD
A  NRAPTLHRLGIQAFEPILVEGRAIQLHPLVCPAFNADFDGD
N  NRAPTLHRLGIQSFEPILVEGRAIQLHPLVCPAFNADFDGD
E  NRAPTLHRLGIQSFQPKVTFEKAILLHPLVCSAFNADFDGD
L  NRAPTLHRLGIQAFQPILVNGRAIHLHPLVCGGFNADFDGD
T  NRAPTLHRLGIQAFQPVLVEGRAICLHPLVCKGFNADFDGD
S  NRAPTLHRLGIQAFQPILVEGRAICLHPLVCKGFNADFDGD
P  NRAPTLHRMNLQSFKPLLTEGYSLKFYPLGCTSFNADFDGD
```

FIG. 1. Alignment of *rpoC* sequences used. The sequences are from *Escherichia coli* (C), *Anabaena variabilis* (A), *Nostoc commune* (N), *Euglena gracilis* (E), Liverwort (*Marchantia polymorpha*, L), Tobacco (*Nicotiana tabacum*, T), Spinach (*Spinacia oleracea*, S) and *Plasmodium falciparum* (P). The sequence corresponds to nucleotides 2400–2447, 2829–2933, 3090–3224 and 3360–3482 of the *P. falciparum* sequence.

placed within the photosynthetic clade, although in this case it was placed within the landplant chloroplast clade. This position for the *Euglena–Plasmodium* clade was not robust, however, as a tree that was otherwise identical but placed it outside the landplant chloroplasts but within the photosynthetic lineage [i.e. the same as that generated by distance matrix analysis, Fig. 2(a)] was not significantly worse at the 5% level. Trees that placed the *Plasmodium* sequence (with or without the *Euglena* sequence) outside a cyanobacterial/landplant chloroplast clade were significantly worse at the 5% level. Similar results were obtained with parsimony analysis of the

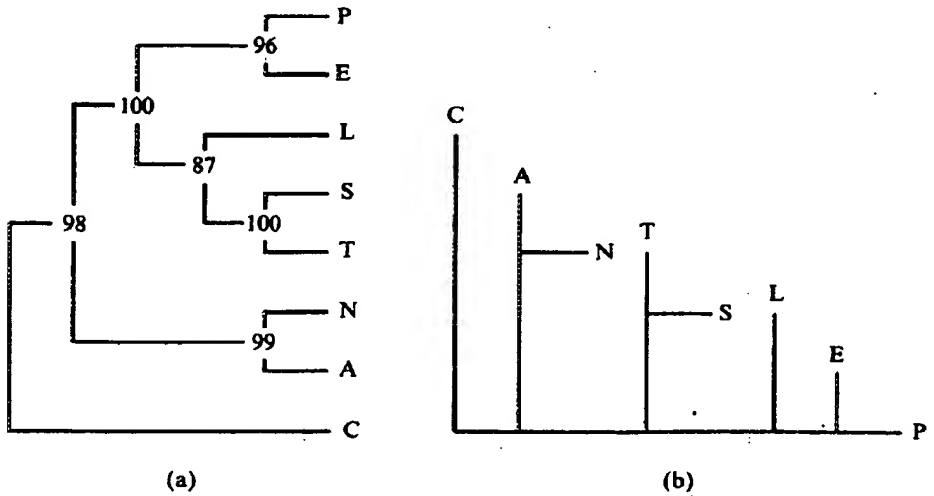

FIG. 2. Inferred "best" trees. The figure shows the best trees obtained with (a) distance matrix and (b) maximum likelihood and parsimony methods. The numbers at the forks of the distance matrix tree indicate the number of times the group consisting of the species which are to the right of that fork occurred out of 100 trees, omiting third position data. Abbreviations are as for Fig. 1. Branch lengths are arbitrary.

nucleotide sequences; the most parsimonious tree was that shown in Fig. 2(b) but the topology shown in Fig. 2(a) was not significantly worse at the 5% level.

There are too many hypothetical topologies with eight taxa to allow all of them to be tested pairwise conveniently, so a subset of the taxa was taken and all possible topologies tested. The *E. coli*, *Nostoc*, *Euglena*, Tobacco and *Plasmodium* sequences were used for this purpose, and all possible topologies (with *E. coli* as an outgroup, shown in Fig. 3) were tested. The results of hypothesis testing using maximum likelihood and parsimony are shown in Table 1. For both methods the best tree placed the *Plasmodium* sequence within the photosynthetic clade and specifically with *Euglena* (Tree 14). All trees that placed the *Plasmodium* sequence outside the photosynthetic clade (Trees 1, 6 and 11) were significantly worse at the 5% level.

These data strongly suggest that the *Plasmodium* sequence originated within the photosynthetic lineage. However, it might be argued that although the bootstrapping and significance testing show this conclusion is robust against random errors, it could have been distorted through systematic errors. The two likely causes of systematic errors are (i) attraction of the branches to *Plasmodium* and *Euglena*, causing the taxa to be grouped artificially closely, because they are relatively long (Felsenstein, 1978) or (ii) the A–T richness of the *Plasmodium* and plastid sequences resulting in homoplasy (independent mutations to the same base at a given site) and consequent grouping of the sequences too closely (Lockhart *et al.*, 1992). To avoid errors caused by differing branch lengths, the *E. coli*, *Nostoc*, *Euglena* and *Plasmodium* sequences were analyzed by the operator invariant method of Lake, which is not affected by this problem (Lake, 1987). The tree grouping *E. coli* with *Nostoc* and *Euglena* with *Plasmodium* was significantly supported; the other two theoretically

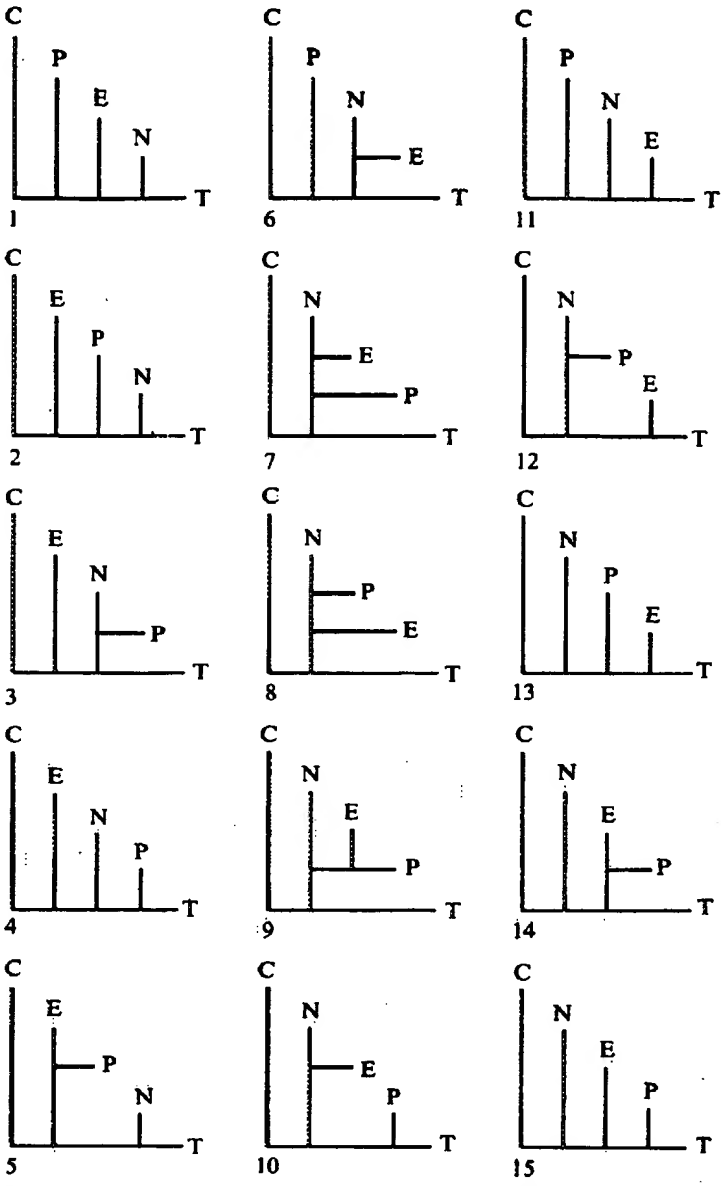

FIG. 3. Tree topologies tested by maximum likelihood and parsimony methods. Abbreviations are as for Fig. 1. Branch lengths are arbitrary.

possible trees (with *E. coli–Plasmodium*, *Nostoc–Euglena* and *E. coli–Euglena*, *Nostoc–Plasmodium* groupings) were not significantly supported (not shown). This result indicates that the association of the *Plasmodium* and *Euglena* sequences does not result from their being "long edges". Distortion of trees through biased substitution patterns ("substitutional bias") can be inferred when tree topologies generated using different codon positions are inconsistent, with strongest evidence for the

TABLE 1

Outcomes of hypothesis-testing under maximum-likelihood (ML) and parsimony (PARS). For each tree topology (as shown in Fig. 3) the table indicates if it is the best (++), not significantly worse than the best at the 5% level (+) or significantly worse (−)

Tree no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
ML	−	−	−	−	−	−	−	−	−	−	−	−	+	++	+
PARS	−	−	−	−	+	−	−	−	+	−	−	−	−	++	+

distorted topology being found at the third position, where substitutional bias is seen most strongly because of the degeneracy of the genetic code (Lockhart *et al.*, 1992). Most parsimonious trees generated with individual codon positions all contained the *Plasmodium–Euglena* grouping, but fewer trees without this grouping were found to be significantly worse at the 5% level using third position data than with first or second position data (not shown). These results indicate that the *Plasmodium–Euglena* clade does not result from substitutional bias.

Taken together, these results suggest that the *Plasmodium* extrachromosomal *rpoC* gene originated within the photosynthetic lineage, sharing most recent common ancestry with (of those sequences tested) the *Euglena* sequence (although this does not *necessarily* mean that the *Euglena* and *Plasmodium* host cells and nuclear genomes are closely related). Results obtained with the *rpoB* gene (not shown) were consistent with this conclusion, although the lack of a suitable region of cyanobacterial *rpoB* sequence made it impossible to distinguish between trees placing the *Plasmodium–Euglena* clade outside the chloroplast clade but within the cyanobacterial–chloroplast clade and those placing it outside the cyanobacterial–chloroplast clade altogether. Analysis of the other sequenced region of the *Plasmodium* 35 kbp extrachromosomal circle also suggests a photosynthetic origin (Gardner *et al.*, 1991).

Two explanations of these observations seem possible. One is that the sequences are non-functional and were acquired by chance lateral transfer from an ingested photosynthetic organism. However, this would not explain the fact that the sequences are retained and highly conserved in a range of different *Plasmodium* species (Kilejian, 1975; Dore *et al.*, 1983; Williamson *et al.*, 1985; Gardner *et al.*, 1988, 1991). The other, arguably more likely, explanation is that the *Plasmodium* species are descended from a photosynthetic ancestor. Although they are no longer photosynthetic, they have retained part of their plastid DNA, presumably in an organelle that is no longer recognizable as a chloroplast and which is present in the cell preparations used for preparation of mitochondrial DNA. Retention of plastid DNA by organisms that are no longer photosynthetic has been reported for flowering plants (Day & Ellis, 1984; de Pamphilis & Palmer, 1990), *Astasia longa* (a flagellate protozoan resembling *E. gracilis*) and bleached mutants of *E. gracilis* (Siemeister & Hachtel, 1989). It has been suggested that the DNA retained is important in the biosynthesis of haem for other compartments of the cell (Howe & Smith, 1991). If the *Plasmodium* extrachromosomal circle is indeed a plastid remnant, it would be predicted that other genes on it will be homologous to those of chloroplasts.

This suggestion is consistent with the inclusion on ultrastructural grounds of *Plasmodium* in the same branch (Miozoa) of the subkingdom Mitozoa of the kingdom Protozoa as the dinoflagellates, which also contain photosynthetic members (Cavalier-Smith, 1987). It will be interesting to see if other currently non-photosynthetic Protozoa retain indications of a photosynthetic ancestry.

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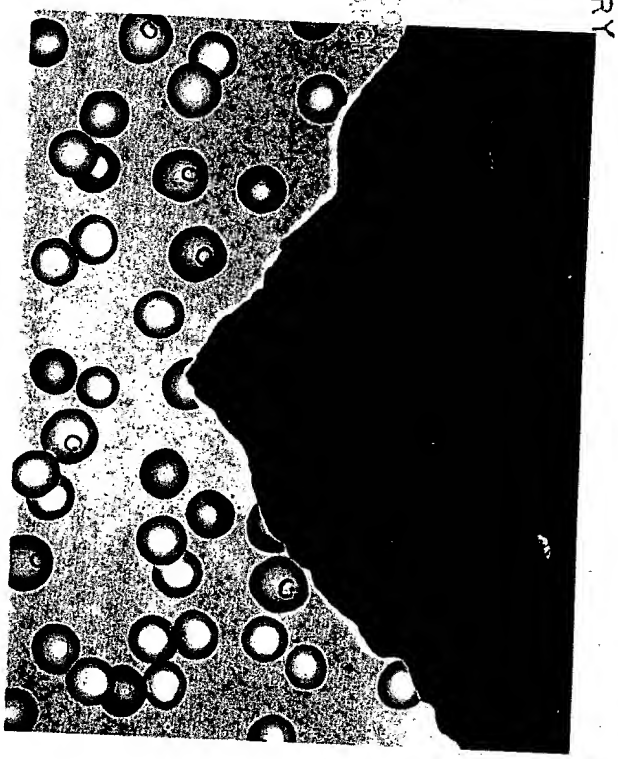

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MOBILE REPEAT UNITS IN *PLASMODIUM BERGHEI*

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Abstract. Repetitive elements (2.3 kb in length), present in tandem arrays in the subtelomeric region of most, but not all, *P. berghei* chromosomes, can be exchanged between non homologous chromosomes and share some properties with mobile elements.

Key words: *Plasmodium berghei*, subtelomeric regions, mobile units, repetitive DNA.

Subtelomeric regions are known to be prone to genomic rearrangements in different lower eukaryotes. In *Plasmodium* species, chromosome-size polymorphism could be mapped in several instances to subtelomeric chromosomal portions. In order to understand the molecular basis of this hypervariability, we undertook a study of the organisation of subtelomeric regions in *Plasmodium berghei*. We had previously achieved cloning and sequencing of plasmodial telomeres (Ponzi *et al.*, 1985; Dore *et al.*, 1986). Telomeric structures, essential for chromosome stabilty, generally consist of an array of simple tandem repeats. In *Plasmodium* species two different repeat versions (GGGATTT and GGGACTT) are irregularly distributed along the telomeric structure, a fact which enabled us recently to study the dynamics of telomere turnover (Ponzi *et al.*, 1992).

The availability of telomeric probes allowed us to continue the exploration of plasmodial chromosomes from the telomere inward. It was thus possible to demonstrate that on most (but not all) *P. berghei* chromosomal ends, the telomere (typically about 1 kb in length) is directly flanked by a repetitive structure, where a 2.1-kb sequence is reiterated and periodically intercalated with a 0.15 kb stretch of telomere-derived sequence (Dore *et al.*, 1990), forming a 2.3-kb modular structure (Fig. 1a).

In turn, internal, telomere-derived stretches contain a characteristic motif: three tandem 27-bp repeats, each of which is comprised of two canonical and two non-canonical telomeric repeats (Pace *et al.*, 1987). These are in a region delimited by inverted repeats as represented in Figure 1b. The same 27-bp motifs are often found also in the innermost part of the telomeric structure (Dore *et al.*, 1990). This kind of organisation very similar to that present in *S. cerevisiae* subtelomeric regions (Chan and Tye, 1983; Walmsley *et al.*, 1984; Zakian and Blanton, 1988) suggests possible mechanisms for chromosome size variation. First of all, it should be noted that internal telomeric tracts may well represent fragile sites. In a fact, a chromosomal break in the vicinity of one of those tracts can be easily healed, through *de novo* addition of telomeric repeats by a telomerase enzymatic activity primed by the newly exposed, telomere-derived motif. Terminal losses would thus be easily tolerated. Secondly, unequal crossing-over events in the common repetitive structure can result in physical exchanges of subtelomeric repeats between non-homologous chromosomes, causing reciprocal size variations.

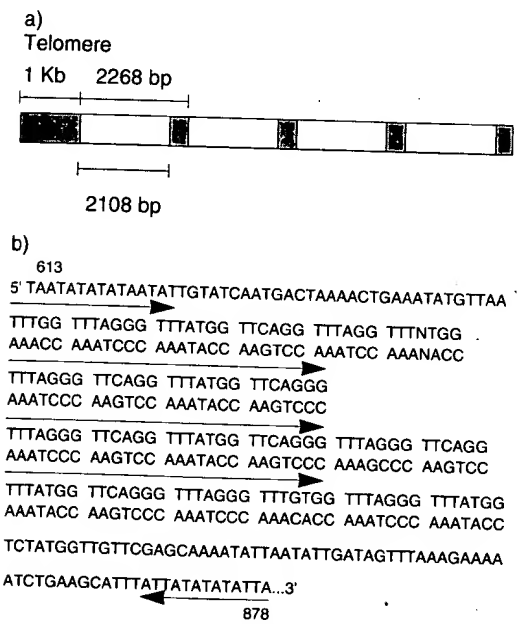

Fig. 1. a) Organization of subtelomeric repeats in *P. berghei*. Shaded areas indicate telomeric or telomere-derived sequences. b) Telomere-derived sequence present in internal telomeric tracts; arrows indicate the three 27-bp motifs and the flanking inverted repeats.

Finally, pairing between an internal telomeric tract and an actual telomere can lead to the insertion of a number of 2.3-kb modules into the telomere itself. This latter kind of event has been directly demonstrated by the experiments described in this parer, showing that 2.3-kb modules are elements capable of displacement from one chromosome to another.

Results and Discussion

Figure 2 gives a schematic presentation of the molecular karyotype (obtained by pulsed field electrophoresis) of: a) a cloned line of *P. berghei* ANKA (8417HP) and, b) a line derived from it by mechanical passages in mice (HPA62). Shadowed rectangles indicate chromosomal bands positive to hybridisation with the 2.3-kb element, used as a probe.

Chromosome 4 in the original clone (lane a) is not lit up by this probe, even under conditions revealing one single copy of the 2.3-kb repeat. The same chromosome (identified by a chromosome-specific probe) in the passaged line has a higher molecular weight and is positive to the 2.3-kb probe (Pace *et al.*, 1990). In order to study the nature and the localisation of the extra DNA present in the enlarged version, both versions of chromosome 4 were recovered from the agarose gel, digested whith rare-cutting enzymes and run in electrophoretical conditions allowing separation of the restriction products. Terminal restric-

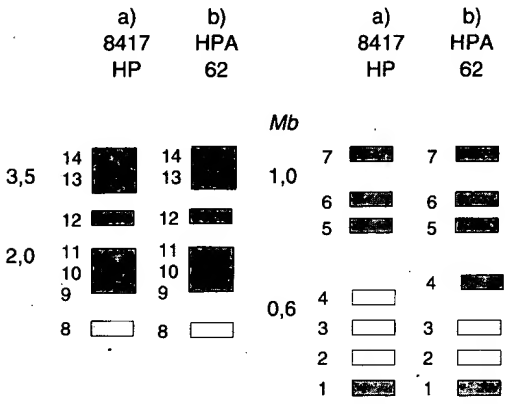

Fig. 2. Molecular karyotypes of *P. berghei* ANKA lines: a) clone 8417 HP (High gametocyte producer) and b) HPA62, obtained from 8417 HP by mechanical passages in mice. Shaded bands indicate chromosomes or chromosome groups containing 2.3-kb elements.

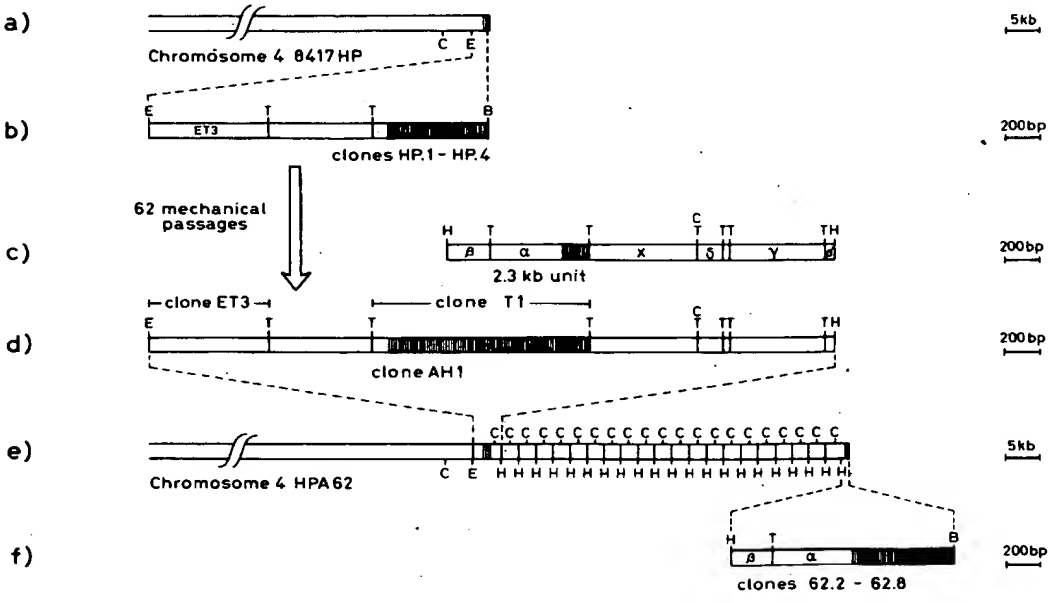

Fig. 3. Schematic presentation of the events modifying one extremity of chromosome 4. In the original version, the telomere is directly flanked by chromosome-specific sequence. After 62 mechanical passages, most of the passaged population (HPA 62) possesses an enlarged version of chromosome 4 (shown in e) on the same scale as in a) containing an insertion which splits the original telomere. The insertion consists of a cluster of tandemly arranged copies of the 2.3-kb element (whose restriction map is given in c); d) and f) show, on an enlarged scale, details of the regions flanking the insertion.

tion fragments (TRFs) were identified by the use of telomeric probe. Of the two TRFs present in the original, smaller version one was not modified in the larger version, while the other exhibited a significant size increase (50-kb).

An accurate study, involving restriction mapping, subcloning and partial sequencing of the modified extremity present in the enlarged version led to the results summarized in Figure 3. The modification was shown to consist in an insertion event which splits the original telomere. The insertion, comprised of more than 20 copies of the 2.3-kb repeats, initiates and ends in correspondance whith the 27-bp motifs. Surprisingly enough, four copies of this motifs, instead of the three usually present, flank the insertion on both sides and in the same orientation.

The insertion leaves about 900 bp of the original telomere on the proximal side as demonstrated by the identity in the sequence of the first 47 telomeric repeats with telomeric clones obtained from the same unmodified extremity (Ponzi *et al.*, 1992). At the distal end, another 600-bp of telomeric sequence builds up the new telomere (Ponzi *et al.*, 1992). These results indicate that a *P. berghei* chromosome completely lacking 2.3-kb repeats can acquire them, most probably as a consequence of a recombinational event involving an actual telomere and an internal telomeric tract. The 27-bp motifs appear to play a definite role in the recombination event mediating the transfer of 2.3-kb units, as they are duplicated in the final structure. It thus appears that 2.3-kb elements can be mobilised as a cluster, flanked by telomere-derived motifs.

Other telomere-bearing, transposon-like elements have been descrved in *Oxytricha* (Herrick *et al.*, 1985) and *Tetrahymena* (Cherry and Blackburn, 1985). At difference with the present case, however, their flanking telomeric sequences are in inverted orientation, as if they had acquired telomeres during an autonomous life in a linear state. This is not the case with the 2.3-kb elements in *P. berghei*, where direct telomeric repeats flank the mobil units.

The ability of *P. berghei* chromosome to expand and contract in units of 2.3-kb modules rather resembles what has been described for the yeast *S. cerevisiae*, where subtelomeric repeated elements (6kb in length), named Y, can be exchanged between chromosomes and also acquired by linear plasmids (Dunn *et al.*, 1984), most probably through a circular intermediate (Horowitz and Haber, 1985). It has also been demonstrated (Henderson and Petes, 1992) that in *S. cerevisiae* internal poly (GT/CA) tracts are able to interact and recombine with yeast-specific telomeric sequences (C1-3 A).

Work is in progress to ascertain the actual mechanism of the *P. berghei* mobile 2.3-kb elements, and to understand the possible biological role of these repeated structures, carefully mantained in mosquito-transmitted lines, but progressively lost in the course of mechanical transmission.

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Tandemly arranged gene clusters of malarial parasites that are highly conserved and transcribed.
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Tandemly arranged gene clusters of malarial parasites that are highly conserved and transcribed

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A molecular clone containing a 5.8 kb *Eco* RI fragment was isolated from a genomic library of the rodent malarial parasite *Plasmodium yoelii*. The *P. yoelii* genome contains about 150 copies of this sequence, making up almost 3% of the DNA. These sequences are tandemly arrayed in head-to-tail configurations with the unit length of the repeat being 5.8 kb. Several poly(A^+) RNAs of *P. yoelii* ranging from 1.6 to 0.3 kb are recognized by the 5.8 kb clone. Five additional species of malarial parasites (*P. chabaudi*, *P. berghei*, *P. falciparum*, *P. knowlesi*, and *P. cynomolgi*) contain tandemly repeated arrays of sequences having the same unit length of 5.8 kb, which readily hybridize to the sequence cloned from *P. yoelii*.

Key words: *Plasmodium*; Malaria; Gene expression; Evolution; Repetitive DNA; Gene organization

Introduction

Molecular genetic investigations of malarial parasites have identified several unique and interesting features of genomic organization. Firstly, all molecularly cloned genes encoding different antigens of plasmodia contain a variety of short, repeated sequences (with varying degrees of conservation for any given gene) within their coding region, specifying tandemly repeated antigenic determinants [1–8]. These sequences vary vastly from one species to the other, or even from one isolate of a species to the other, for any given analogous antigen. Secondly, pulsed-field gradient (PFG) gel electrophoretic analyses of the *Plasmodium falciparum* genome suggest dramatic alterations in the sizes of different chromosomes, which in some instances result from the loss of a large proportion of a given chromosome [9–11]. Thirdly, guanosine + cytosine (G+C) contents of DNA from certain groups of malarial parasites (e.g., rodent vs. primate) differ substantially from each other, and sequences encoding highly conserved housekeeping proteins such as actin and tubulins appear to have markedly diverged between these groups [12]. The picture that emerges from these data is of organisms that exhibit an unusual degree of genetic plasticity.

All eucaryotic genomes contain highly reiterated sequences, some of which may be considered nothing more than 'selfish' DNA [13,14], whereas others, such as tandemly arrayed rRNA genes, play a pivotal role in the survival of the organism [15]. The former demonstrate substantial genetic drift suggesting a lack of any evolutionary pressures to maintain their exact nucleotide sequence, whereas other sequences are well-conserved. Like other eucaryotes, *P. falciparum* contains species-specific repeated sequences [16–18], some of which are clusters of tandemly arranged 21 base-pair imperfect and variable repeats [19,20]. In this report, we describe a 5.8 kb sequence cloned from *P. yoelii* that is tandemly repeated about 150 times in the genome and is

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Abbreviations: PFG, pulsed field gradient; TNE, Tris-NaCl-EDTA; SDS, sodium dodecyl sulfate; SSC, standard saline-citrate; kb, kilobase pairs.

transcribed into poly(A^+) RNAs. At least five other highly diverse malarial parasite species contain homologous tandemly repeated clusters of sequences which have the same unit length of 5.8 kilobase pairs (kb). When seen in the light of the variation in the G+C content and some of the housekeeping genes of these species, this conservation of a transcribed gene cluster may indicate an important role for these tandem arrays in the biology of malarial parasites.

Materials and Methods

Parasites. Stabilates of *Plasmodium yoelii* 17XL, *P. chabaudi*, and *P. berghei* (NYU-2) were originally obtained from Dr. J. Finerty, NIH. Four- to 8-week old BALB/c mice from our breeding colony were inoculated with approximately 10^5 parasites intraperitoneally; at the time of ascending parasitemia, these donor mice were exsanguinated, and 10^6 parasitized red blood cells were injected into a new set of mice to obtain large numbers of parasites. *P. falciparum* (FCR-3 knobless strain) was grown by the in vitro culture system of Trager and Jensen [21].

DNA purification. The anesthetized mice were bled via retro-orbital puncture into an isotonic Tris-buffered saline solution (TNE: 0.05 M Tris-HCl, pH 7.4, 0.1 M NaCl, 0.01 M EDTA) containing 10 IU heparin ml^{-1}. After the removal of buffy coat and defibrination by shaking with glass beads, contaminating leucocytes and platelets were removed by two consecutive passages through columns of microcrystalline cellulose [22]. The erythrocyte fraction was centrifuged, and the pellet was resuspended in 10 volumes of 10 mM Tris-HCl, pH 8.3, 50 mM EDTA, and 1% sodium dodecyl sulfate (SDS). DNA was purified by treatments with RNase A, proteinase K, phenol-chloroform extractions, and ethanol precipitation. Concentrations and purity of DNA were determined by spectrophotometry. The above procedure was used for the purification of *P. yoelii* DNA used in construction of genomic libraries. For other parasite species, a similar procedure was used except that the steps involving cellulose columns were omitted. DNA samples from *P. knowlesi* and *P. cynomolgi* were kindly provided by Dr. T. McCutchan.

RNA purification. RNA was extracted from parasitized mouse blood by a modified procedure involving guanidine isothiocyanate followed by precipitations with sodium acetate and ethanol, as described previously [23,24]. Poly(A^+) RNA was obtained by batch chromatography on oligo(dT) cellulose [25].

Probes. Nick-translation [26] in the presence of [α-^{32}P]dCTP was used to obtain radioactive DNA probes from total *P. yoelii* DNA, pPyαT2 (a plasmid with a 6.6 kb insert containing a 3′ portion of α-tubulin gene of *P. yoelii* (Arasu and Vaidya, in preparation)), and pPy5.8 (see below). The specific activities of the probes were about 1–2 $\times$ 10^8 cpm μg^{-1}.

Agarose gel electrophoresis and blot analyses. DNA was digested with various restriction enzymes purchased from either New England Biolabs or Bethesda Research Laboratories. The digests were electrophoresed on 0.8% agarose gel and transferred to nitrocellulose by the technique of Southern [27]. RNA was subjected to formaldehyde-agarose gel electrophoresis [28] and transferred to nitrocellulose by the technique of Thomas [29]. The blots were prehybridized at 42°C in a buffer containing 50% formamide, 5 $\times$ Denhardt's solution, 3 $\times$ standard saline citrate (SSC: 0.15 M NaCl, 0.015 M sodium-citrate), 200 μg ml^{-1} yeast RNA, 50 μg ml^{-1} denatured calf thymus DNA, and 50 mM Tris-HCl, pH 7.4. Hybridizations were carried out in the same buffer after the inclusion of the denatured radioactive probe (0.5–1 $\times$ 10^6 cpm ml^{-1}). After the hybridization, blots were washed for 5 min with 2 $\times$ SSC, 0.1% SDS, at room temperature, followed by three 20 min washes with 0.1 $\times$ SSC, 0.1% SDS at 50°C. Kodak X-Omat AR film was exposed to air-dried blots in the presence of intensifying screens at −70°C, and developed in an automated film processor.

Recombinant phage library. P. yoelii DNA digested to completion with *Eco* RI was mixed with *Eco* RI–*Bam* HI–*Sal* I-digested DNA of phage vector λL47.1 [30]. After ligation, the DNA was

packaged in vitro [31], and the library was amplified in the *E. coli* host strain BHB2600. This genomic library was screened with the nick-translated total *P. yoelii* DNA by the method of Benton and Davis [32]. The 5.8 kb *Eco* RI fragment was subcloned into pBR322 and propagated in *E. coli* strain HB101.

Results

Molecular cloning and restriction map of a 5.8 kb P. yoelii DNA. A phage λL47.1 library containing *Eco* RI fragments of *P. yoelii* was constructed and screened with nick-translated ^{32}P-labelled total *P. yoelii* DNA. As expected, a large number of plaques contained sequences recognized by this probe, and four such plaques were randomly picked and purified. DNA was isolated from these four recombinants, digested with *Eco* RI, Southern blotted, and hybridized with nick-translated total *P. yoelii* DNA probe. Three of the four recombinant phage contained a 5.8 kb *Eco* RI fragment hybridizing to this probe (data not shown). One of these phage (called λPy5.8) was then chosen for further analysis, and the 5.8 kb fragment from it was inserted into the *Eco* RI site of pBR322 to give the plasmid pPy5.8. A restriction map of the 5.8 kb insert is shown in Fig. 1. The enzymes *Bam* HI, *Pst* I, *Xho* I, *Sal* I, *Kpn* I, and *Sst* I failed to cleave within the insert of pPy5.8.

Estimating the copy number of the 5.8 kb sequence. A highly repeated sequence has been cloned from *P. falciparum* [19,20] and has been shown to be dispersed throughout the genome,

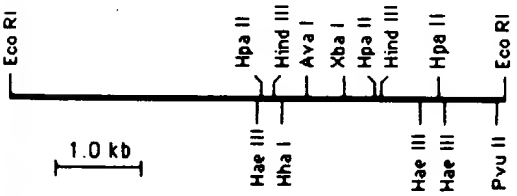

Fig. 1. Restriction endonuclease map of the 5.8 kb unit cloned from *P. yoelii*. The map was constructed from single and double digestions of pPy5.8 with various restriction endonucleases and measurements of the sizes of ethidium bromide stained fragments. Confirmations were also obtained by hybridization of Southern blots of these gels with the nick-translated probe from the total *P. yoelii* DNA.

which is reminiscent of repeated sequences of many other eucaryotic cells. If the pPy5.8 insert contained within it a similarly dispersed sequence, a probe from it would hybridize to many different fragments of *P. yoelii* DNA, resulting in a smear of hybridization after the Southern blot analysis. Instead, a very prominent 5.8 kb band was observed in Southern blots of *Eco* RI-digested *P. yoelii* DNA probed with pPy5.8. This also provided a means to estimate the copy number of this sequence in the *P. yoelii* genome. As shown in Fig. 2, varying amounts of *Eco* RI-digested *P. yoelii* DNA were electrophoresed, and the Southern blots were hybridized to probes synthesized from either pPy5.8 or pPyαT2, a plasmid carrying a 6.6 kb *Eco* RI fragment of *P. yoelii* DNA with the 3′-end region of an α-tubulin gene (Arasu and Vaidya, in preparation). Both these probes were ^{32}P-labelled with equivalent specific activity (1×10^8 cpm μg^{-1}), and were from fragments of comparable size; in addition, the 6.6 kb fragment bearing α-tubulin sequence is present as a single copy in the *P. yoelii* genome (Arasu and Vaidya, unpublished results). Densitometric comparison of hybridization bands obtained with these two probes showed that the intensity of the 5.8 kb band obtained when 60 ng of *P. yoelii* DNA was hybridized to pPy5.8 was about 8.6-fold greater than the intensity of the 6.6 kb band obtained when 1000 ng of the same DNA was probed with pPyαT2. It should be pointed out that these data could be influenced by variations in the A+T contents of the two probes and/or the sequence organization within the 5.8 kb insert. However, our sequencing data on *P. yoelii* α-tubulin gene suggest approximately 68% A+T content (Arasu and Vaidya, in preparation) which is comparable to other genes of malarial parasites; furthermore, in the absence of any sequence information on the 5.8 kb insert, it is not possible to correct for the bias that may be introduced by a putative difference in the A+T content of the probes. Nonetheless, given these caveats, we estimate that there are approximately 150 copies of the 5.8 kb sequence in *P. yoelii* DNA; that is, 870 kb of *P. yoelii* DNA consists of members closely related to this sequence. Assuming a genome size of about 30000 kb for malarial parasites, this sequence family could account for about 3% of the *P. yoelii* genome.

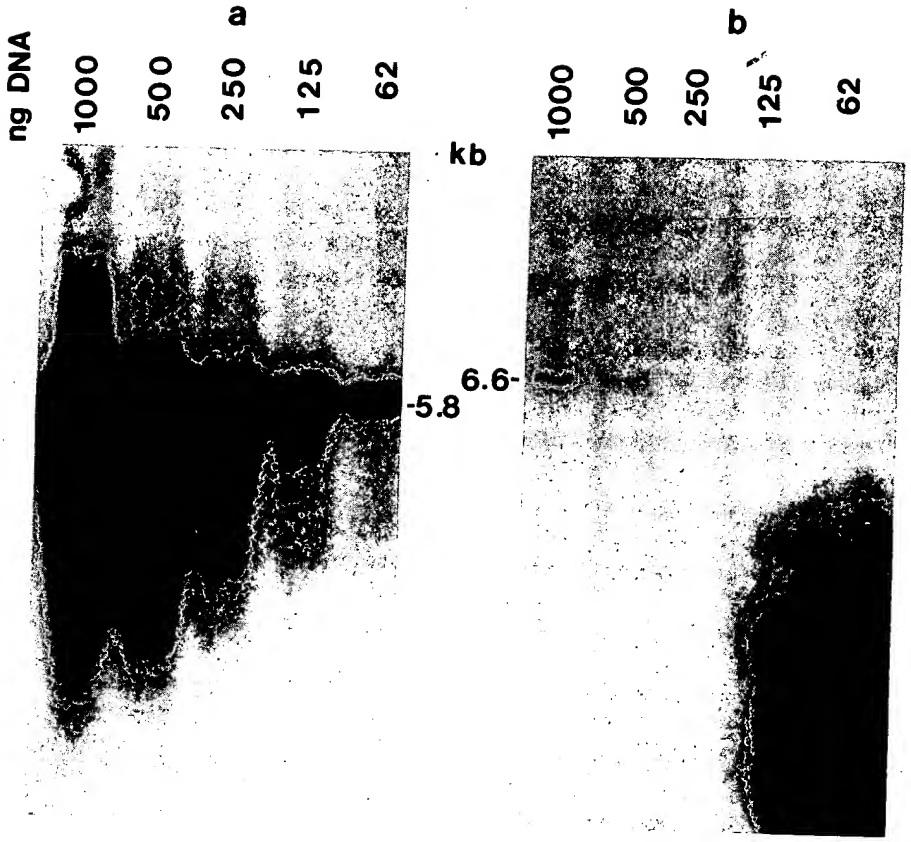

Fig. 2. Estimation of the copy number for the 5.8 kb sequence in *P. yoelii* DNA. DNA purified from *P. yoelii* 17XL was digested with *Eco* RI, and the indicated amounts (in two sets of lanes) were electrophoresed in 0.8% agarose gel transferred to a nitrocellulose sheet. The total amount of DNA in each lane was kept constant by including calf thymus DNA as a carrier. The nitrocellulose sheet was divided into two parts. One part (a) was hybridized to a nick-translated probe synthesized from the plasmid pPy5.8; the other part (b) was hybridized to a probe synthesized from a clone (pPyαT2) carrying the 3′ portion of an α-tubulin gene of *P. yoelii* (Arasu and Vaidya, manuscript in preparation). Both the probes were of comparable specific activity (1×10^8 dpm μg^{-1}). Radioautographic exposure was for 6 h in the presence of intensifying screens. Densitometric scanning of the prominent bands in each radioautograph was used to estimate the copy number of the 5.8 kb sequence in *P. yoelii*.

Organization of the 5.8 kb sequences in the P. yoelii genome. The 5.8 kb sequence family could be present as either: (i) unintegrated molecules; (ii) parts of a larger sequence family that is dispersed throughout the genome; or (iii) as tandemly repeated cluster(s). In distinguishing between these possibilities, we took advantage of the restriction map of pPy5.8 (Fig. 1). The first possibility was eliminated when several restriction enzymes that do not cleave the 5.8 kb sequence left high molecular weight smears in Southern blot hybridizations of genomic DNA (e.g., *Bam* HI digestion in Fig. 3). A tandemly and directly repeated cluster of identical sequences would give a circularly permuted map when digested with restriction enzymes that cleave more than once within the repeating unit. For example, *Hind* III cleaves twice within the pPy5.8 insert (Fig. 1), dividing it into 3.2 kb, 1.35 kb, and 1.25 kb fragments. In genomic DNA digestions, if the 5.8 kb sequence was tandemly arrayed, *Hind* III cleavage would give 4.45 kb (3.2+1.25 kb) and 1.35 kb fragments, and *Hind* III + *Eco* RI double cleavage would give 3.2, 1.35, and 1.25 kb fragments. As shown in Fig. 3, this is precisely what was observed. Hence most, if not all, of the 5.8 kb sequences are tandemly and directly repeated. PFG electrophoresis blots of a number of different

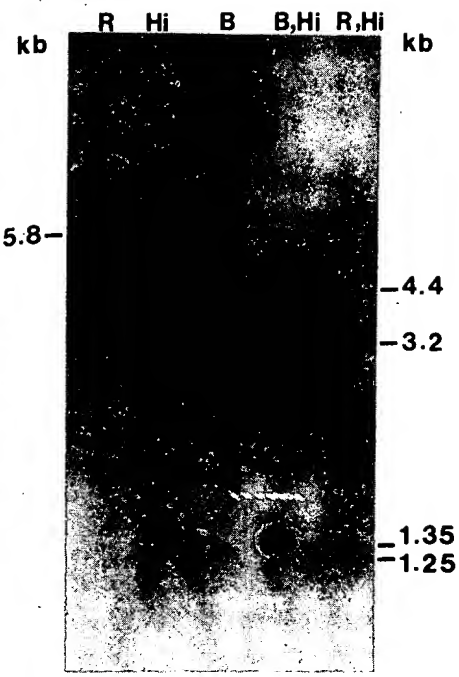

Fig. 3. Circularly permuted restriction digestion bands from *P. yoelii* DNA hybridizing to pPy5.8, indicating tandem and direct repetition of the 5.8 kb sequences. *P. yoelii* DNA was digested with *Eco* RI (R), *Hind* III (Hi), *Bam* HI (B) or doubly digested (either B+Hi or R+Hi), electrophoresed, and Southern blotted. Hybridization was with the nick-translated pPy5.8. The sizes of the fragments are indicated at the right.

plasmodial species when hybridized to the pPy5.8 probe revealed the presence of these clusters on those fragments of parasite genome that fail to enter such gels (K. Suplick, H. Rubin and A. Vaidya, unpublished results). This indicates that either the tandem 5.8 kb clusters are all present on very large chromosomes or in nicked circular DNA molecules that fail to enter PFG gels. Within the sequences that make up these clusters, there is relatively little divergence, as indicated by the conservation of restriction sites in all the members of the family; the restriction map of pPy5.8 clone appears to be valid for essentially all other 5.8 kb sequences of *P. yoelii*.

Transcription of the 5.8 sequences. RNA extracted from the blood of mice infected with *P. yoelii* was analyzed by Northern blotting for the presence of transcripts homologous to pPy5.8, as shown in Fig. 4. Total mouse RNA (from mammary glands), total *P. yoelii* RNA, and poly(A$^+$) *P. yoelii* RNA were probed with pPy5.8. A number of transcripts, apparently containing tracts of poly(A) (cf. Fig. 4b and 4c), were recognized by the pPy5.8 probe. The sizes of transcripts were estimated to be 1.6 kb, 1.15 kb, 1.0 kb, 0.7 kb, and a strongly hybridizing region of $\leq$ 0.3 kb. The smallest RNA molecules detected by the probe were not the result of degradation of RNA, since the same RNA, when hybridized to the *P. yoelii*

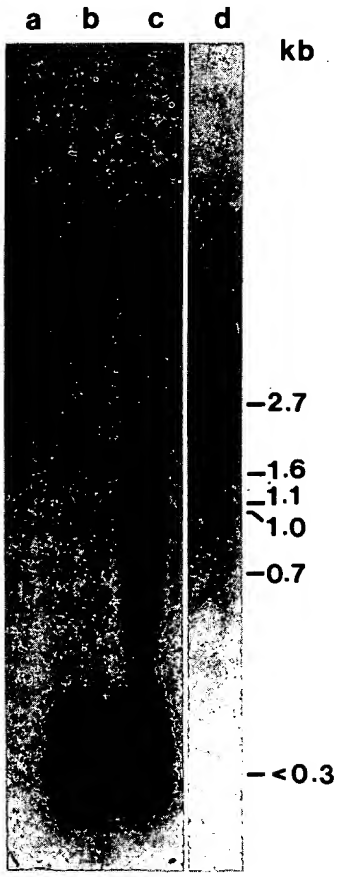

Fig. 4. Poly(A$^+$) RNA homologous to pPy5.8 in *P. yoelii*-infected erythrocytes. RNA extracted from the blood of mice infected with *P. yoelii* 17× as well as mouse mammary glands was subjected to denaturing formaldehyde agarose gel electrophoresis and transferred to a nitrocellulose sheet. Hybridization was with either the pPy5.8 probe (lanes a–c) or an α-tubulin probe (lane d) from *P. yoelii*. Lane a: 20 μg mouse mammary gland RNA; lane b: 20 μg total *P. yoelii*-RNA; lane c: 2 μg poly(A$^+$) RNA from *P. yoelii*; lane d is the same as lane c except that the probe was from an α-tubulin clone. The numbers represent the size of various transcripts.

α-tubulin probe, gave a single 2.7 kb band with no indication of degradation (Fig. 4d). Pre-boiled RNase A treatment of the samples prior to Northern blot analysis completely eliminated hybridizing nucleic acids (not shown), proving that the sequences detected were not degraded DNA molecules.

Evolutionary conservation of the 5.8 kb sequence. We examined DNA from five other species of malarial parasites for the presence of sequences hybridizing to pPy5.8. DNA was isolated from two rodent parasites, *P. berghei* and *P. chabaudi adami*, two primate parasites, *P. knowlesi*, and *P. cynomolgi*, and the human parasite *P. falciparum*. Southern blot analysis of digestion of these DNA samples with various restriction enzymes is shown in Fig. 5. Under relatively stringent conditions, every malarial parasite species tested was found to contain sequences homologous to the *P. yoelii* 5.8 kb clone. Most interestingly, these sequences were present as tandemly repeated arrays with an invariant unit length of 5.8 kb. This conclusion was based upon the observation that in each case, there were two different restriction enzymes giving a unit length 5.8 kb hybridizing band; this is possible if all the 5.8 kb sequences are directly and tandemly repeated. In the case of *P. cynomolgi*, another enzyme besides *Ava* I that cleaves only once within the cluster is not shown; however, the smaller bands produced by *Hpa* II add up to 5.8 kb, thereby supporting the circularly permuted map of these tandem arrays. Although we have not estimated the copy number of the 5.8 kb sequence in each of these species, the intensity of the hybridization bands appears to be comparable to those seen in Southern blots of *P. yoelii* DNA, suggesting a comparable repeat frequency in all the species tested.

We have also examined DNA from *Trypanosoma brucei*, *Saccharomyces cerevisiae*, and mice for the presence of sequences homologous to pPy5.8. No hybridization was detectable, even under nonstringent conditions (data not shown).

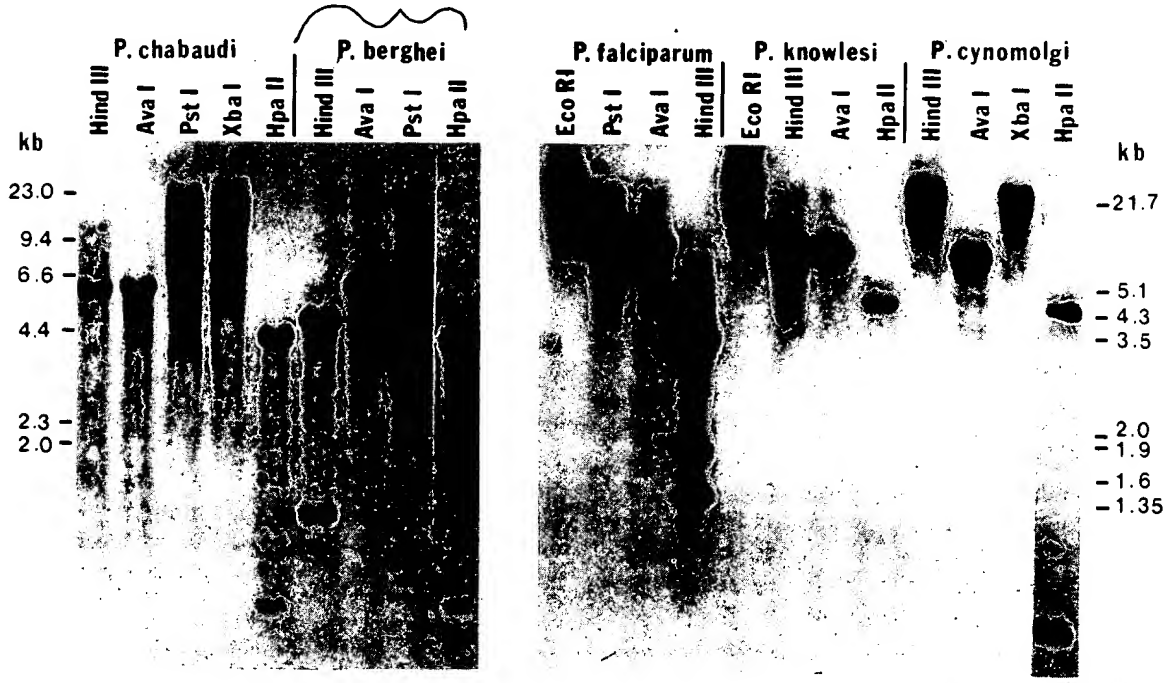

Fig. 5. Five highly diverse malarial parasite species contain arrays of tandemly repeated 5.8 kb sequence recognized by pPy5.8. DNAs from *P. chabaudi*, *P. berghei*, *P. falciparum*, *P. knowlesi*, and *P. cynomolgi* were digested with the indicated restriction enzymes, subjected to agarose gel electrophoresis, and blotted onto nitrocellulose sheets. The blots were hybridized with nick-translated pPy5.8 probe under relatively stringent conditions.

This suggests that the 5.8 kb sequence cluster is unique to malarial parasites.

Discussion

In this paper, we have described a 5.8 kb sequence of malarial genome that is tandemly and directly repeated, making up almost 3% of the DNA in *P. yoelii*. Tandemly arrayed genes are not uncommon among eucaryotes; histone genes in sea urchins [33] and rRNA genes in most eucaryotes [16] are prime examples of such arrays and are believed to be important in providing an adequate supply of transcripts from these genes through a gene-dose effect. In trypanosomatid protozoa, tubulin genes are present in tandem arrays [34,35], as are sequences encoding the spliced-leaders or mini-exons present on all mRNAs of these organisms [36]. Although tandemly repeated short sequences specifying the repeated epitopes of most plasmodial antigens have been described by several groups [1–8], to our knowledge, this is the first report of a long DNA sequence of malarial parasites that is tandemly repeated in a manner reminiscent of the examples cited above. However, the 5.8 kb sequence does not appear to encode histones, rRNA, α-tubulins, β-tubulin, or actin as determined by hybridization with heterologous (histone H4, β-tubulin, and actin) or homologous (rRNA and α-tubulin) probes under conditions that did detect DNA fragments other than those derived from the 5.8 kb clusters (data not shown). Furthermore, a probe from the 5.8 kb clone does not hybridize to any sequences in trypanosomes, yeast, or mice, suggesting that, at least at the DNA level, this sequence is unique for malarial parasites.

At least five distinct RNA molecules in the blood stages of *P. yoelii* ranging in size from ≤ 0.3 to 1.6 kb were recognized by the 5.8 kb probe. These RNAs had tracts of poly(A) and may represent mRNAs unless their purification on oligo(dT)-cellulose was fortuitous due to a high A content. Strongly hybridizing RNA molecules of ≤ 0.3 kb are reminiscent of the 135 nucleotide-long spliced-leader precursor RNA molecules observed in trypanosomes [37,38]. However, unlike the trypanosome system where every mRNA has a common leader sequence derived from the 135-base precursor, only a few distinct mRNA bands were detected in *P. yoelii*. Hence, we cannot as yet ascribe the role of spliced-leader donor to the plasmodial 5.8 kb cluster in a manner analogous to the trypanosomes. RNA molecules of ≤ 0.3 kb as well as of larger sizes hybridizing to the pPy5.8 probe have also been observed in *P. falciparum* (Vaidya, unpublished observations), indicating that the transcription of the 5.8 kb clusters is not limited only to *P. yoelii*. The apparent abundance of the transcripts homologous to the 5.8 kb cluster was comparable to that of the α-tubulin mRNA. However, this did not correspond to the abundance of the gene cluster, which amounted to almost 3% of the entire genome. It is possible that in some other stages of malarial parasite development transcripts from the 5.8 kb clusters may be much more numerous than observed in the blood stages.

The most remarkable observation was the detection of 5.8 kb tandemly arrayed clusters in every malarial parasite tested. Although it is difficult to estimate evolutionary distances between different species of malarial parasites, it is believed that certain primate parasites such as *P. knowlesi* and *P. cynomolgi* are evolutionarily much more distant from rodent parasites and *P. falciparum*. This is reflected in the G+C content of the DNA as well as the apparent divergence of usually highly conserved genes such as actin and tubulin in these species [12]. In spite of this, the 5.8 kb sequence from each of these species appears to have extensive homology as judged by cross-hybridization under relatively stringent conditions. Moreover, the size of the repeating unit is remarkably constant over this evolutionary distance. Genes encoding many of the surface antigens of malarial parasites undergo rapid sequence changes [1–3,7,39–41]. Furthermore, PFG electrophoresis of *P. falciparum* chromosome-length DNA indicates almost routine genomic deletions and rearrangements, even among individual clones of a single isolate [9–11]. Amid this sea of apparent genetic plasticity lie islands of the 5.8 kb clusters which are highly stable over a long evolutionary period. We do not know the function of these clusters, but we believe it must be vital, for it can tolerate little deviation. While the

biological function of these tandem arrays would be most interesting to elucidate, their relative abundance in all tested malarial parasites certainly points to the possibility of a molecular diagnostic probe capable of detecting many malarias.

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TITLE: Intact gene and method of excising and cloning same

Drawing Description Text (5):
FIG. 3 is a map of the plasmid pPbSL7.8 insert and shows restriction sites as well as 3 mung bean clevage sites which occur in restrictions containing 45% formamide. The thick lines represent, from left to right, the coding area for the small rRNA, the 5.8S RNA and 2.2 kb of the large rRNA of P. berghei. FIG. 3A Mung bean nuclease sites in a cloned fragment of the ribosomal gene of P. berghei. 0.5 .mu.g aliquots of pPbSL7.8 DNA were treated with EcoRI (lane 1), mung bean nuclease and EcoRI sequentially (lane 2) or mung bean nuclease alone (lane 3). Products from reactions were then electrophoresed through 1.2% agarose at 2 v/cm for 18 hrs, and the DNA visualized by staining with ethidium bromide. FIG. 3B The comparision of digestion products resulting from mung bean cleavage of cloned and genomic DNA. DNA from pPbSL7.8 (lane 1), and .lambda.Pb27 (lane 2) were digested with mung bean nuclease as described herein. Genomic DNA (5 .mu.g) was digested with mung bean nuclease in 40% (lane 3) or 45% (lane 4) formamide. The products were compared by Southern blot analysis using a radiolabelled plasmid (pPbSL5.6) probe which contains only the gene for the small rRNA subunit. The band at 6.7 kb in lane 1 results from hybridization of the probe to a pBr322 derived sequence on the blot.

Detailed Description Text (22):
It should be noted that cleavage depends on the structure of the naked DNA. A cloned DNA sequence synthesized in and isolated from E. coli yields the same cleavage products as genomic DNA from Plasmodium. Mung bean nuclease cleavage of cloned Plasmodium ribosomal genes in formamide yields fragments of defined size which correspond to the coding areas for the small ribosomal RNA, the 5.8S RNA and the large ribosomal RNA. FIG. 3 shows a map of a cloned plasmid, pPbSL7.8, which contains an EcoRI DNA fragment from P. berghei with the coding region for the entire small rRNA, the 5.8S RNA and 2.2 kb of the large ribosomal gene which is interrupted by an EcoRI cleavage site. The data in FIG. 3A show the products of cleavage of the cloned restriction fragment with either a restriction nuclease or mung bean nuclease. Mung bean nuclease cleavage products of cloned ribosomal genes are directly compared with those from total DNA in FIG. 3B. The major Plasmodium derived fragments are all the same size. This suggests that mung bean nuclease cuts these cloned Plasmodium DNAs nearly quantitatively and yields the same products as genomic DNA. The fact that both cloned and genomic DNAs react identically indicates that the DNA has not been previously cut by Plasmodium nucleases. Therefore, cleavage depends on the structure of the DNA clone. DNA from other organisms were also analyzed. Cleavage that yields fragments of defined size was not uncommon even in DNA from higher eukaryotic organisms (FIG. 1A, lane 3). Some DNAs, like that of the parasitic trematode Schisotosoma mansoni, yielded single, small fragments that hybridized either to actin or tubulin probes. This indicates broader utility of the present invention.